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COMPARATIVE EFFECTS OF GLUCAGON, HYDROCORTISONE AND EPINEPHRINE ON THE PROTEIN METABOLISM OF THE FASTING RAT[1,2]

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ABSTRACT

An approach toward the elucidation of the mechanism of action of glucagon in promoting protein catabolism has been made by comparing the metabolic effects of glucagon and hydrocortisone using female rats fasted five days. The possible relationship of the level of liver glycogen to protein catabolism has also been investigated by comparing the action of glucagon and epinephrine.

Both glucagon and hydrocortisone-treated animals lost more weight and excreted more urinary nitrogen, phosphorus and creatinine than controls. The rate of nitrogen and phosphorus excretion of glucagon-treated rats declined progressively whereas that of hydrocortisone-treated rats increased progressively throughout the course of the experiment. Although the animals in both groups lost similar amounts of urinary nitrogen the glucagon-treated rats lost less body weight, tissue protein and urinary phosphorus. Furthermore, hydrocortisone caused an elevation in the level of blood α amino nitrogen whereas glucagon caused blood α amino nitrogen to be depressed. These results suggest that glucagon and hydrocortisone cause protein catabolism by different mechanisms. It is proposed that glucagon acts directly on the liver to facilitate the catabolism of amino acids in contrast to the postulated action of hydrocortisone in mobilizing amino acids from extrahepatic tissues.

Epinephrine and glucagon depressed liver glycogen but the glycogenolytic effect of epinephrine was of borderline significance. However, epinephrine-treated rats did not differ from controls in respect to any of the above mentioned parameters of protein metabolism. This suggests that the protein catabolic action of glucagon is not directly a consequence of reduced hepatic glycogen.

REPEATED intramuscular injections of glucagon to optimally regulated diabetic subjects have been shown to cause not only a temporary aggravation of the diabetes but also a loss of body nitrogen, (1, 2,

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[2] Presented in part at the Federation Meetings, April 1958.

3). Furthermore, the increased nitrogen excretion could not be satisfactorily explained on the basis of the elevated levels of blood and urinary glucose *per se*. This suggested that the increased protein catabolism was a direct action of glucagon and not merely a consequence of the aggravated diabetic state. This conclusion is supported by reports that in the fasted rat glucagon administration causes increased nitrogen excretion without producing glucosuria or changes in blood sugar (4, 5). Increased nitrogen excretion following glucagon administration has also been observed in the rabbit (6) and in the dog (5).

As an approach toward characterizing more precisely the nature and significance of the protein catabolic action of glucagon it seemed worthwhile to compare the protein catabolic actions of glucagon and hydrocortisone in the fasting rat. A simultaneous comparison of the effects of glucagon and epinephrine on protein and carbohydrate metabolism also seemed pertinent in an attempt to define the relationship between hepatic glycogen content and protein catabolism. To these ends, the three hormones have been compared in the fasting rat on the basis of their ability to influence the excretion of nitrogen, phosphorus and creatinine as well as from the standpoint of their influence on the content of glycogen and protein of liver and muscle. The effects of the hormones on the levels of blood glucose and amino acids have also been compared.

MATERIALS AND METHODS

Young adult female rats of a Wistar strain were selected from stock when 145–160 gm. in weight. Animals were randomly distributed among the various experimental groups and were individually housed in metabolism cages with only water available during the course of the experiment.

The following experimental groups were established: 1) Control Group; rats in this group received 0.1 ml. peanut oil twice daily. 2) Glucagon Group; animals in this group received 0.75 mg. of crystalline glucagon in 0.1 ml. peanut oil two times a day. 3) Epinephrine Group; the rats in this group received 0.05 mg. of epinephrine in 0.1 ml. oil twice daily. 4) Hydrocortisone Group; the animals in this group received 0.25 mg. of hydrocortisone in 0.1 ml. oil two times a day. All injections were given subcutaneously, alternating among neck, right flank and left flank. Rats were weighed and urine was collected daily.

On the morning of the sixth day (12 hours after the last injection) after five full days of fasting the animals were anesthetized with 0.05 ml./100 gm. body weight of pentobarbital (60 mg./ml.). Ten minutes after the injection of the pentobarbital blood was drawn by cardiac puncture into oxalated syringes and tubes, and the various tissues were removed.

Tissue glycogen was determined by the anthrone method of Seifter *et al.* (7). Analyses were made of the diaphragm, right gastrocnemius and an aliquot of the lower right quadrant of the liver. These tissues were rapidly dissected and transferred to previously tared centrifuge tubes containing 3 ml. of cold 30% KOH. After reweighing the sample size was determined by difference. Samples were hydrolyzed and brought to appropriate volumes after repeated precipitations with 95% ethanol. After dissection of the samples

for glycogen determination the left gastrocnemius, kidneys, adrenals and the remainder of the liver were removed and weighed.

The water content of liver and left gastrocnemius was determined by drying in a 95° C oven to constant weight. Analyses for total nitrogen of the dried samples of these organs, as well as of the daily urine collection and the pooled five day fecal sample were carried out by a modification of the micro-Kjeldahl method (8).

Daily urine samples were analyzed for phosphorus by the method of Fiske and Subba-Row (9), and for creatinine by a modification of the Jaffe reaction (10). Urinary glucose was evaluated daily by the method of Somogyi (11).

Aliquots of cardiac blood were analyzed for glucose by Somogyi's technique (11) and amino nitrogen was assayed according to Frame, Russell and Wilhelmi (12).

The animals withstood five days of fasting remarkably well. The rats were alert and in apparent good health at the time of sacrifice. In the rare instance in which the animal appeared moribund it was discarded from the experiment. It may be worth noting that the epinephrine treated rats were more restive, upon handling, than the other animals.

RESULTS

Urinary Nitrogen. A summary of the data concerning urinary nitrogen is shown in Figure 1. The glucagon-treated rats excreted much greater average quantities of nitrogen (5.70 gm.) than the fasted controls (3.65 gm.) per kilogram of body weight over the five day fasting period. The observed differences in mean daily nitrogen excretion between the two groups were highly significant ($P < .01$) for the first four days of fasting, but the differences were not significant ($P > .05$) for the fifth day. It may be pertinent to point out that the control rats excreted more nitrogen on the last day of fasting than on the second, third, and fourth days. In contrast, the epinephrine-treated animals did not excrete significantly ($P > .05$) more nitrogen than the controls over the five day fasting period (Fig. 1).

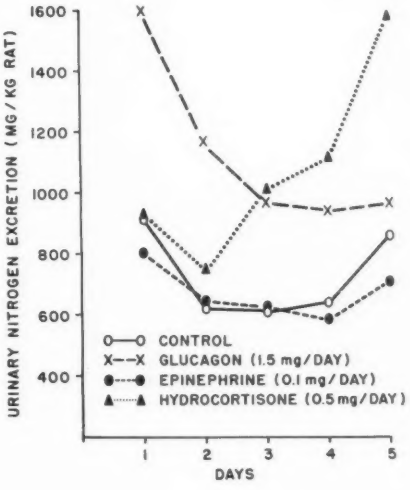

FIG. 1. Influence of glucagon, hydrocortisone and epinephrine on the urinary nitrogen excretion of fasting rats. The standard error for any value in the figure is approximately 62.0.

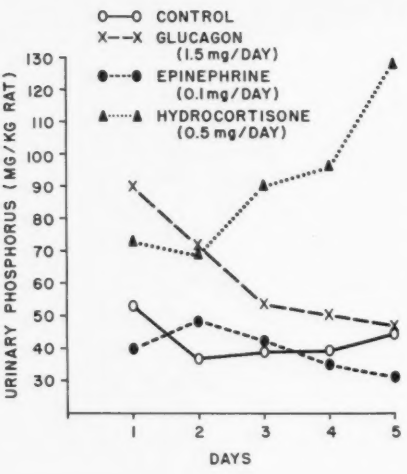

FIG. 2. Influence of glucagon, hydrocortisone and epinephrine on the urinary phosphorus excretion of fasting rats. The standard error for any value in the figure is approximately 1.7.

As would be expected, the rats that were given hydrocortisone also excreted more nitrogen than the controls (Fig. 1). Although the total amount of nitrogen that was excreted by the hydrocortisone group during the five day fasting period was of the same order of magnitude (5.45 gm./kg.) as that of the glucagon group, the patterns of nitrogen excretion of the two groups were strikingly different. The increased nitrogen excretion of the glucagon-treated rats was maximal at the outset and tended to wane progressively as fasting continued. However, the increased nitrogen excretion induced by hydrocortisone increased progressively as the fasting continued so that the greatest excretion of nitrogen was reached on the fifth day.

Urinary Phosphorus. In general, the patterns of mean daily excretion of phosphorous (Fig. 2) for the controls, glucagon-, hydrocortisone- and epinephrine-treated groups of rats reflected fairly closely the corresponding changes in urinary nitrogen excretion. An increase in urinary phosphorus excretion over that of controls accompanied the elevated nitrogen excretion of the glucagon- and hydrocortisone-treated rats. The observed difference between the control and glucagon-treated rats was significant only on days 1 and 2 of the fast, whereas the differences between the control and the hydrocortisone-treated rats were significant only on days 3, 4 and 5 of the fasting period. The animals receiving hydrocortisone excreted significantly more phosphorus than the glucagon group. The total phosphorus excretion for the 5 day fasting period amounted to 217 mg./kg. for the controls, 314 mg./kg. for the glucagon rats, and 460 mg./kg. for the hydrocortisone-treated animals.

Urinary Creatinine. The rats treated with glucagon, hydrocortisone or

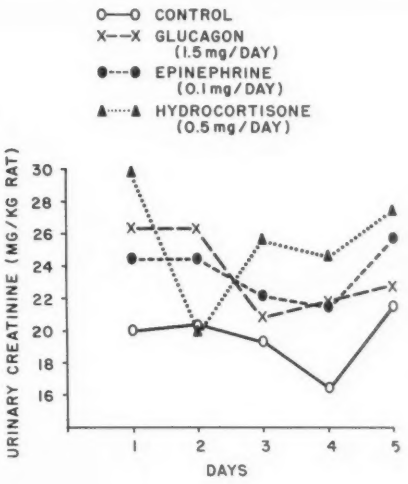

FIG. 3. Influence of glucagon, hydrocortisone and epinephrine on the urinary creatinine excretion of fasting rats. The standard error for any value in the figure is approximately 1.6.

epinephrine excreted significantly greater amounts of creatinine daily than the control group during the course of the experiment (Fig. 3). However, unlike the phosphorus patterns the mean daily patterns of creatinine excretion did not closely parallel the corresponding daily changes in nitrogen excretion.

Blood α-Amino Nitrogen. Glucagon and hydrocortisone appeared to have diametrically opposed effects on the level of blood amino acids (Table 1). At the end of the 5-day fasting period, the mean level of blood α-amino nitrogen of the glucagon-treated rats was reduced to approximately two-thirds of the level of the control animals, whereas the mean level of blood α-amino nitrogen of the hydrocortisone-treated animals was increased by slightly more than a third above control levels. The observed differences were highly significant ($P < .01$). In contrast, no difference was noted in the levels of blood α-amino nitrogen between the rats that were given epinephrine and the control rats.

TABLE 1. INFLUENCE OF VARIOUS HORMONES ON THE MEAN BLOOD LEVELS OF GLUCOSE AND α-AMINO ACID N IN FEMALE RATS STARVED FOR FIVE DAYS[1]

Group	No. of animals	Mg. %	
		Blood glucose	Blood amino N
Control	10	88.5	9.8
Glucagon	11	89.1	6.9
Epinephrine	10	50.6	9.3
Hydrocortisone	7	35.7	13.5
Standard Error for Column Entry		6.0	0.6

[1] The standard error in each case is of the form $s/\sqrt{\bar{n}}$ where s is a pooled estimate of the intra-group standard deviation (based on 34 degrees of freedom) and $\bar{n} = 9.5$ is the average group size.

Body Weight, Organ Weight and Tissue Protein (Table 2). The rats that received glucagon or hydrocortisone during the 5-day fasting period lost respectively 14.5% and 26.8% more weight than the controls, whereas the animals that were treated with epinephrine showed no significantly different weight loss from that of the control group.

The mean weights of the liver, gastrocnemius muscle and adrenals of the glucagon-treated animals were significantly less than those for the controls ($P < .05$). Administration of hydrocortisone was associated with a significant reduction in weight of diaphragm as well as of the gastrocnemius muscle. Neither glucagon, hydrocortisone nor epinephrine had any effect on kidney weight. There were no significant changes in the content of water in either the liver or the gastrocneumius muscle of the epinephrine or glucagon-treated animals. However, the water content of both liver and gastrocnemius muscle was significantly elevated in the rats treated with hydrocortisone.

The protein concentrations in the livers and gastrocnemius muscles of the glucagon-treated animals did not differ significantly from those of the controls. However, treatment with epinephrine resulted in significant depressions of both liver- and muscle-protein concentration. Hydrocortisone decreased the per cent protein of the liver but not of the gastrocnemius.

The total protein contents of the livers and gastrocnemius muscles of the animals receiving either glucagon or hydrocortisone were calculated to be significantly less than those of the controls. This could be attributed in large part to the decreased weight of the above mentioned organs (*vide supra*). On the other hand, the total protein content of the liver and gastrocnemius muscle was not altered by administration of epinephrine.

Blood Glucose. The mean levels of blood glucose of the glucagon-treated rats at the end of five days fast were not different from those of the control animals (Table 1). Administration of epinephrine or hydrocortisone resulted in a significant lowering of the blood glucose levels. The reduction was more marked in the case of hydrocortisone.

Tissue Glycogen. In comparison with control animals, the hepatic glycogen

TABLE 2. INFLUENCE OF VARIOUS HORMONES ON BODY WEIGHT, ORGAN WEIGHT AND TISSUE PROTEIN OF FEMALE RATS STARVED FIVE DAYS[1]

Group	No. rats	Body wt. (gm.)		Organ weight					% H_2O		% protein[2]		Total protein (mg.)	
		Start	Loss	Liver (gm.)	Diaphragm (mg.)	gastrocnemius (mg.)	adrenal (mg.)	kidney (gm.)	Liver	Gastrocnemius	Liver	Gastrocnemius	Liver	Gastrocnemius
Control	10	156	41	3.56	310.2	650.4	36.5	1.054	72.71	76.34	75.82	86.38	735.2	132.9
Glucagon	11	154	48	3.01	297.6	579.2	31.2	1.056	73.06	76.94	75.43	85.37	608.9	114.1
Epinephrine	10	159	44	3.65	264.5	672.1	37.8	1.086	72.99	75.95	72.56	81.88	711.9	132.4
Hydrocortisone	7	148	52	3.31	272.5	491.9	32.9	1.074	77.38	77.81	71.99	87.72	541.9	95.6
Standard Error for Column Entry			1.6	0.16	11.7	25.4	1.8	0.039	0.65	0.59	0.60	0.59	16.1	2.7

[1] See footnote Table 1.
[2] Protein analysis on a dry weight basis.

TABLE 3. THE INFLUENCE OF VARIOUS HORMONES ON THE MEAN GLYCOGEN CONCENTRATION OF DIAPHRAGM, LIVER AND GASTROCNEMIUS OF FEMALE RATS STARVED FOR FIVE DAYS[1]

Group	No. of animals	% glycogen		
		Diaphragm	liver	Gastrocnemius
Control	10	0.264	0.681	0.387
Glucagon	11	0.247	0.302	0.339
Epinephrine	10	0.229	0.476	0.364
Hydrocortisone	7	0.372	0.907	0.343
Standard Error for Column Entry		0.024	0.099	0.034

[1] See footnote Table 1.

levels of the glucagon-treated rats were reduced by 56%; this is a significant difference (Table 3). Epinephrine administration resulted in a 31% reduction of hepatic glycogen from control values, whereas hydrocortisone administration was associated with a 33% elevation in liver glycogen content; but these changes are of borderline significance. Hydrocortisone treatment of the starved rat resulted in a significant increase in the percentage of glycogen in the diaphragm but neither epinephrine nor glucagon had any effect on the diaphragmatic glycogen content. None of the experimental procedures appeared to alter the glycogen concentration of gastrocnemius muscle.

DISCUSSION

The results clearly show that glucagon was capable of stimulating protein catabolism in the starved rat just as it did in the fed animal (5), or in the fed-insulin-treated diabetic subject (1–3). However, the hyperglycemia and glycosuria which have been characteristically associated with the repeated administration of glucagon were absent. This is in agreement with the results of other workers (4, 5). The accelerated breakdown of body protein was evident not only from the increased excretion of urinary nitrogen, phosphorus and creatinine but also from the greater loss in body weight and decreased mass of liver and muscle tissues of the glucagon-treated rats compared to the controls.

The protein catabolic action of glucagon and hydrocortisone differed in several noteworthy respects. Glucagon caused a decrease while hydrocortisone caused an increase in the level of circulating amino acids. The increase in nitrogen and phosphorus excretion induced by glucagon administration was maximal at the onset and tended to decrease progressively as the fasting continued, whereas the extra nitrogen excretion induced by hydrocortisone increased progressively, attaining the highest value on the last day of the fast.

It is of interest that although the total amount of nitrogen that was excreted by the hydrocortisone group during the five day fasting period was of the same order of magnitude as that of the glucagon group, the

hydrocortisone-treated rats exhibited a greater loss of muscle mass as well as a greater loss in body weight. Furthermore, the phosphorus excretion of the hydrocortisone animals was significantly greater than that of the glucagon animals.

The differences in the above parameters suggest that the mechanism of protein catabolism of glucagon is different from that of hydrocortisone, and hence is probably not mediated by a stimulation of adrenal cortical secretions. Furthermore, the differences provide some insight into the locus and nature of the protein catabolic action of glucagon.

It has been postualted that adrenal cortical hormones act mainly outside of the liver when they affect nitrogen metabolism serving to facilitate the mobilization of amino acids (13) and tissue protein (14), by promoting protein catabolism and inhibiting protein anabolism (15). This concept has been based largely on the following type of experimental evidence. An increase in the level of plasma amino acids has been observed following the administration of either adrenal cortical extract (ACE) (16) or ACTH (17, 18) to intact or eviscerated animals. Conversely, adrenalectomy has been shown to lower the rate of accumulation of plasma amino acids both in eviscerated (19, 20) and non-eviscerated (16) rats, while the administration of glucose has been reported to abolish the extra amino acid accumulation after ACE treatment in the eviscerated rat (20). Experiments *in vitro* (21) have also shown that the amino nitrogen production from the diaphragms of adrenalectomized rats is significantly less than normal and may be restored to normal by pretreatment of the animals with ACE. Our findings in the intact, fasted, hydrocortisone-treated rat are in agreement with these observations and lend support to the hypothesis just outlined for the mechanism of protein catabolism by hydrocortisone. By the same line of reasoning the fall in blood amino acids in the face of increased urea production and nitrogen excretion may be considered as presumptive evidence that in the glucagon-treated rats, amino acids are being removed and deaminated by the liver at an accelerated rate. This suggests that the locus of action of glucagon in nitrogen metabolism is predominantly hepatic and at the amino acid level, facilitating gluconeogenesis from amino acids.

The decreased loss of body weight and tissue mass of the glucagon animals as compared to the hydrocortisone animals even though both groups excreted approximately the same amount of extra nitrogen over that of the controls suggests that the extra nitrogen excreted by the animals receiving glucagon may have been derived from the available pool of free amino acids as well as from the breakdown of tissue protein.

The failure of epinephrine to depress the level of blood α-amino nitrogen or to increase protein catabolism despite an apparent glycogenolytic effect argues against the probability that the influence of glucagon in increasing the rate of gluconeogenesis from amino acids is a result of its glycogen-

lytic action. However, it should be pointed out that our results are at variance with those of Rose and Nelson (22), who did show an effect of epinephrine on protein catabolism when the hormone was given in sufficiently large amounts to depress liver glycogen. These investigators demonstrated an accumulation of urea in the blood of nephrectomized rats as well as a decrease in liver glycogen following intraportal, but not systemic, injections of 7 μg/100 gm. rat/hour of epinephrine. The significance of the glycogen changes in the studies of Nelson and Rose is equivocal in view of the very low values of liver glycogen of their control groups.

In view of the increased hepatic glycogen content and increased protein catabolism of the hydrocortisone-treated rats we did not anticipate the low blood sugar values that we observed in this group of animals. Nor would we have expected that treatment with epinephrine, a hyperglycemic, glycogenolytic agent, would have resulted in the observed low blood sugar values. We have no explanation for the causal factors leading to this change in blood sugar. It should be noted however, that the animals in both groups tolerated the five day fast extremely well. None of the animals were moribund.

Although the precise relationship between the glycogenolytic and protein catabolic actions of glucagon remains to be defined, the observed influences of glucagon on nitrogen metabolism in the fed and in the fasted animal, suggest that both actions may be a reflection of a more general effect of the hormone in the liver. The studies thus far are of interest in that they raise the possibility that glucagon, as well as hydrocortisone, is an important hormonal factor in the physiological regulation of protein catabolism. They also raise the possibility that glucagon may be an important hormonal link between carbohydrate and protein metabolism.

ADDENDUM

Since this paper was written, W. C. Shoemaker and T. B. Van Itallie, *Endocrinology*, **66**: 260, 1960 have reported an increased hepatic uptake of alpha amino nitrogen after glucagon-induced glycogenolysis, beginning promptly and reaching a maximum about 45 minutes after glucagon administration. They have interpreted these changes however, to mean increased gluconeogenesis as a consequence of depletion of liver glycogen stores.

Acknowledgments

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REFERENCES

1. Izzo, J. L., A. Roncone and M. A. Paliani: *Fed. Proc.* **16**: 200. 1957.
2. Izzo, J. L. and A. Roncone: *Diabetes* **6**: 45. 1957.

3. Izzo, J. L. and A. Roncone: *Annals N. Y. Academy of Sciences* **74:** 582. 1959.
4. Kalant, N.: *Proc. Soc. Exper. Biol. & Med.* **86:** 617. 1954.
5. Salter, J. M., I. W. F. Davidson and C. H. Best: *Diabetes* **6:** 248. 1957.
6. Tyberghein, J.: *Arch. intern Physiol.* **6:** 104. 1953.
7. Seifter, S., S. Dayton, B. Novic and E. Muntwyler: *J. Biol. Chem.* **25:** 191. 1950.
8. Pregl, F. and H. Roth: Die Quantitative Organische Mikroanalyse, Springer, J., Berlin, Sixth Edition, 1949.
9. Fiske, C. H. and Y. SubbaRow: *J. Biol. Chem.* **66:** 375. 1925.
10. Jaffe, M.: *Z. Physiol. Chem.*, **10:** 391. 1886.
11. Somogyi, M.: *J. Biol. Chem.* **160:** 69. 1946.
12. Frame, E. G., J. A. Russell, A. E. Wilhelmi: *J. Biol. Chem.* **149:** 255. 1943.
13. Engel, F. L.: Recent Progress in Hormone Research. Academic Press Inc., New York, N. Y., 9: 277, 1951.
14. Roberts, S.: *J. Biol. Chem.* **200:** 77. 1953.
15. Hoberman, H. D.: *Yale J. Biol. and Med.* **22:** 344. 1950.
16. Friedberg, F. and D. M. Greenberg: *J. Biol. Chem.* **168:** 405. 1948.
17. Li, C. H., I. Geschwind and H. M. Evans: *J. Biol. Chem.* **177:** 91. 1949.
18. Lotspeich, W. D.: *J. Biol. Chem.* **185:** 221. 1950.
19. Ingle, D. J., M. C. Prestrud and J. E. Nezamis: *Proc. Soc. Exper. Biol. & Med.* **67:** 321. 1948.
20. Bondy, P. K.: *Endocrinology* **45:** 605. 1949.
21. Kline, D. L.: *Endocrinology* **45:** 596. 1949.
22. Rose, S. and J. Nelson: *Australian J. Exper. Biol.* **34:** 33. 1956.

FACTORS INFLUENCING *IN VITRO* SYNTHESIS OF PROGESTERONE[1,2]

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ABSTRACT

Pituitary homogenates from gilts at different stages of the estrous cycle and gestation did not stimulate or inhibit *in vitro* progesterone synthesis by gilt luteal tissue in Krebs-Ringer bicarbonate buffer medium containing 200 mg. % glucose. Non-pituitary gonadotrophins, PMS and HCG; relaxin; and oxytocic materials, Syntocinon and Purified Oxytocic Principle, had no effect on *in vitro* synthesis of progesterone by swine luteal tissue. Ovine lactogenic hormone (prolactin, uteotrophic hormone or LTH) had no detectable effect on *in vitro* or *in vivo* luteal function. Subtotal hysterectomy prolonged the functional life of gilt corpora lutea as indicated by progesterone content and capacity of tissue to synthesize the hormone *in vitro*. Endometrial filtrates from days 12 and 13 of the estrous cycle increased *in vitro* progesterone synthesis by luteal tissue whereas filtrates from days 16 and 18 showed a definite inhibitory effect on hormone production.

RESULTS of experimental work with several species of animals indicate *in vitro* activity of steroid synthesizing tissues can be altered by the inclusion of certain hormones in incubation media. For example, Tepperman (1) has reported that corticotrophin added to slices of dog adrenal cortex caused a reduction in ascorbic acid and an increase in respiration. Production of corticoids by rat adrenal slices, according to Saffran *et al.* (2), was dependent upon the quantity of corticotrophin in the medium. Brady (3) demonstrated that chorionic gonadotrophin favored *in vitro* conversion of acetate to testosterone by slices of rabbit, swine and human testes. According to Hollander and Hollander (4), the conversion of testosterone to estradiol in dog ovary slices was stimulated by FSH.

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Since it has been demonstrated that endocrine organs respond to trophic hormone stimulation under *in vitro* conditions, the objective of this study was to evaluate factors which may influence *in vitro* progesterone synthesis by swine luteal tissue in an attempt to elucidate the yet unknown factors responsible for the development, maintenance and regression of the corpus luteum. The following substances were investigated: homogenates of whole gilt pituitary glands, ovine lactogenic hormone (prolactin, luteotrophic hormone or LTH), gonadotrophic hormones (PMS and HCG), oxytocin, relaxin and gilt striated muscle and uterine filtrates. In addition, data are also presented on effect of subtotal hysterectomy on progesterone content of luteal tissue.

MATERIALS AND METHODS

Animals used in this study were crossbred gilts, weighing 225–300 lbs., which were checked daily with vasectomized boars to determine occurrence of estrus and length of estrous cycles. The average length of estrous cycles in gilts is 21 days and pregnancy is 113 days. Procedures for evaluating *in vitro* progesterone synthesis by gilt luteal tissue and for quantitative estimation of progesterone have been described in detail by Duncan *et al.* (5). This method involves incubating slices of luteal tissue, obtained from gilts immediately following slaughter, in Krebs-Ringer bicarbonate buffer medium containing 200 mg. % gluclose for 2 hours at 37.5° C under an atmosphere of 95% O_2 and 5% CO_2. Following incubation, progesterone content of the tissue and its respective medium is quantitatively estimated by a method utilizing 95% ethanol extraction, aluminum oxide column adsorption chromatography, counter current distribution, paper partition chromatography and ultraviolet absorption spectrophotometry. Reported progesterone concentrations have been adjusted for an average recovery of 84.7%. Determinations were carried out with 550 to 700 mg. of luteal tissue with the exception of day 4 of cycle in which case 150 mg. was used.

Effect of gilt pituitary on *in vitro* synthesis of progesterone by luteal tissue was investigated by incubating whole gland homogenates in Krebs bicarbonate medium with luteal tissue from the same animal. Effects of including the following in incubation medium were investigated: lactogenic hormone (LTH, Armour and Co.) in amounts from 5 to 600 I.U./gm. of luteal tissue; PMS (Gonadogen, The Upjohn Co.) 50 to 2000 I.U./gm.; HCG (Follutein, E. R. Squibb and Co.) 50 to 3800 I.U./gm.; relaxin (Releasin, Warner-Chilcott Laboratories) 60 to 160 μg./gm.; oxytocin (Syntocinon, Sandoz, Inc.) 3 to 112 U.S.P. units/gm.; and (Purified Oxytocic Principle (P.O.P.), Armour and Co.) 1 to 88 U.S.P. units/gm.

Endometrium from gilts at known stages of the reproductive cycle was also prepared as incubation medium. This material, obtained by scraping uterine cornu removed at time of slaughter, was subjected to repeated freezing and thawing (3×). These scrapings were filtered through double layered cheesecloth and the resulting filtrate adjusted to pH 7.4 and buffered with 1.3% sodium bicarbonate prior to use. Depending upon the stage of the reproductive cycle, a single uterus furnishes 20 to 100 ml. of filtrate with approximately 86 mg. of dry matter per ml. Aqueous filtrates of myometrium and striated muscle were prepared for incubation medium in the same manner as endometrium.

Subtotal hysterectomy (from mid-cervix to tubo-uterine junctions) of gilts was performed 7 and 15 days after estrus. Corpora lutea present at time of hysterectomy were marked with carbon black to aid in evaluating their survival. Animals were sluaghtered 25 or 50 days after surgery and initial as well as post 2 hour incubation progesterone concentrations in luteal tissue were determined.

TABLE 1. EFFECT OF HOMOGENIZED GILT PITUITARY AND OF LACTOGENIC HORMONE* ON *In Vitro* PROGESTERONE SYNTHESIS BY GILT LUTEAL TISSUE

Day	No. of animals	Mean progesterone concentration, μg/gm.			
		Initial	After 2 hr. incubation		
			Control	With homogenized pituitary	With lactogenic hormone
4C†	3	21	156	162	156
8C	3	40	112	114	111
12C	4	61	137	131	137
16C	3	74	145	145	144
18C	3	0	0	0	0
16P‡	3	82	121	109	112
24P	4	84	152	140	153
48P	4	105	139	142	143
72P	4	68	106	107	104
96P	3	52	98	133	117
102P	3	21	68	68	68

* Armour and Co. LTH AVLO210.
† Estrous cycle.
‡ Pregnancy.

RESULTS AND DISCUSSION

Table 1 presents data showing effects of homogenized gilt pituitary and lactogenic hormone on *in vitro* synthesis of progesterone by luteal tissue. These data indicate that addition of homogenates of fresh whole pituitary glands from gilts at different stages of the cycle and gestation did not significantly ($P = .05$) inhibit or stimulate synthesis of progesterone by luteal tissue under the experimental procedures employed.

It has been demonstrated that lactogenic hormone activity of gilt pituitaries increases during the luteal phase of the cycle as well as during gestation (6). However, there is no experimental evidence indicating lactogenic hormone has a luteotrophic function in swine. Ovine lactogenic hormone had no significant effect on the intrinsic capacity of luteal tissue slices to synthesize progesterone under the conditions employed (Table 1). Other experiments suggest ovine lactogenic hormone is also ineffectual in altering *in vivo* luteal function in swine. For example, 2 gilts were given intramuscular injections of 125 and 250 I.U. daily for 3 days prior to slaughter on day 12 of the cycle. This treatment did not alter initial concentration of progesterone in luteal tissue or influence *in vitro* synthesis of progesterone. In addition, 2 gilts were given intramuscular injections of lactogenic hormone at levels of 125 and 250 I.U. beginning at day 12 of the cycle and continuing for 13 days. Both animals showed estrus at day 21 indicating this treatment did not extend the functional life span of the corpora lutea. These findings of an apparent lack of luteotrophic action by lactogenic hormone are similar to those reported by Smith *et al.* (7) for the cow. Sammelwitz and Nalbandov (8) found lactogenic hormone failed to counteract ovarian regression in pregnant gilts induced by daily injection of 400 mg. of proges-

terone. Bradbury *et al.* (9) and Hisaw (10) failed to maintain corpora lutea of women and monkeys, respectively, with lactogenic hormone. Rats and possibly sheep (11) are the only animals in which lactogenic hormone has thus far been found to have a luteotrophic activity.

Addition of the non-pituitary gonadotrophins, PMS and HCG, considered luteotrophic in the mare and woman respectively, to incubation medium did not alter *in vitro* synthesis of progesterone by swine luteal tissue. Relaxin, a hormone present in swine luteal tissue, was also without effect. However, the effect of the original level of relaxin present in tissue may have been such as to mask action of additional relaxin. There is experimental evidence suggesting oxytocin may play a part in development of the corpus luteum. Armstrong and Hansel (12) found daily administration of natural or synthetic oxytocin to post-puberal non-lactating heifers during the first week of the estrous cycle resulted in a marked shortening of the diestrual period. These investigators concluded the observed effects of oxytocin were caused by inhibition of normal corpus luteum function, possibly by interfering with secretion of a luteotrophic hormone from anterior pituitary. Addition of chlorbutanol-free Syntocinon (26 to 112 U.S.P. units/gm.) did not alter *in vitro* progesterone synthesis by luteal tissue. Inhibition observed with standard Syntocinon and P.O.P. preparations was caused by their chlorbutanol (0.5%) content. Daily intramuscular or intravenous injection of P.O.P. into gilts for 7 days beginning at estrus, at levels of 5, 25, 75 and 225 U.S.P. units per 100 lbs. body weight, did not alter the diestrual period of treated animals. They returned to estrus approximately 21 days after initial injection. These findings suggest a species difference with respect to the action of oxytocin on life span of corpora lutea in swine and cattle.

According to Asdell (13), growth of the uterus may maintain the corpus luteum while if uterine degeneration occurs for any reason, degeneration of the corpus luteum may follow. The physiologic nature of this dependency is unknown. Proliferation of uterine endometrium in the gilt begins at estrus and attains a maximum about the 8th to 10th day of the cycle. However, during the interval following day 15 to the beginning of the next cycle there is a slow reversion to resting type of all structures. These morphologic changes of endometrium can be correlated with functional development and retrogression of corpora lutea as indicated by the progesterone concentration in luteal tissue (5).

Effects of endometrial filtrates on *in vitro* synthesis of progesterone by luteal tissue are presented in Figure 1. Results are expressed as percentages of the progesterone synthesized by an aliquot of the same tissue incubated in Krebs-Ringer bicarbonate buffer medium. There was an increase in hormone synthesis by tissue from both cycling and pregnant animals when incubation medium was prepared from endometrium from the active luteal phase of the cycle (Days 12 and 13). Significant increases ranged

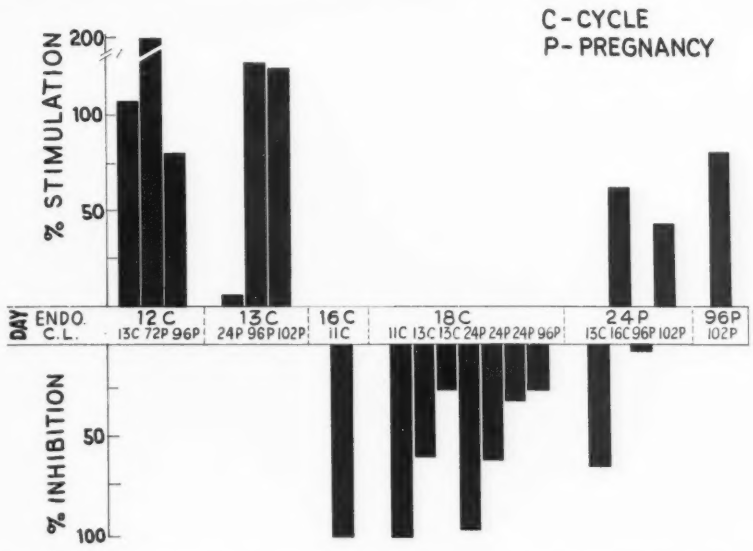

FIG. 1. Effect of endometrial filtrates on *in vitro* progesterone synthesis of gilt luteal tissue as compared to synthesis in Krebs-Ringer bicarbonate buffer medium. Endo. = Endometrium. C. L. = Corpora Lutea.

from 81 to 206% above controls. Medium prepared from endometrium from the late diestrual phase (Days 16 and 18) had a definite inhibitory effect on progesterone synthesis. Results with endometrium from pregnant animals were less conclusive. For example, incubation medium prepared from endometrium at day 24 of gestation showed both significant inhibitory and stimulatory effects. Material prepared from an animal 96 days pregnant increased progesterone synthesis by 81%.

Effect of dilution, dialysis, protein precipitation, heating and solvent extraction of endometrial preparations was studied to obtain preliminary characterization of the factor(s) responsible for these activities. Specific activities of two Day 13 and of five Day 18 filtrates were removed by dialysis against distilled water for 48 hours at 5° C. Inhibition equivalent to untreated endometrial filtrate was observed using as substrate the lyophilized dialyzate obtained following dialysis of late diestrual endometrium for 48 hours. Endometrium capable of 60% inhibition of progesterone synthesis had no effect on synthesis when it was used as medium following dialysis for 48 hours. Aliquots of late diestrual endometrial filtrate heated at 28° C, 50° C, 75° C and 100° C for 15 minutes reduced synthesis by 59%, 43%, 19% and 50%, respectively. With exception of samples heated at 100° C, in which there was considerable denatured protein, inhibitory effect is reduced following heating. Heating active luteal

phase endometrial filtrates at 80° C for ½ hr. completely removed the stimulatory effect. Extent of inhibition observed with late diestrual filtrates was related to the amount of filtrate included in incubation media maintained at a constant total volume of 1 ml./100 mg. tissue with Krebs bicarbonate buffer. Aliquots (5 to 7 ml.) of endometrial filtrates prepared from uteri at Days 13 and 18 of the cycle were ashed. These residues were reconstituted in Krebs bicarbonate buffer. Progesterone synthesis was inhibited up to 90% by preparations from both stages. Aqueous endometrial filtrates were extracted 3× with 10:1 volumes of 95% ethanol. This ethanolic phase was evaporated to dryness *in vacuo* and taken up in Krebs bicarbonate buffer. Two such endometrial preparations from Day 13 of the cycle did not alter *in vitro* synthesis whereas eight preparations from late diestrual material inhibited synthesis. This inhibitory effect was retained following ether extraction of the alcoholic residues under acidic, basic or neutral conditions.

Aqueous myometrial filtrates used as incubation media had the same relative effects as the respective endometrial preparations but of lesser magnitude when compared on a filtrate dry weight basis. Aqueous extracts of striated muscle obtained from 7 gilts during active luteal and late diestrual phases of the cycle consistently inhibited *in vitro* synthesis more than 90%. Inhibitory activity of striated muscle paralleled that of late diestrual endometrium in all of the above characterizations. Following extraction of samples from two late diestrual gilts by the method of Tenny *et al.* (14), inhibitory activity associated with endometrium was found in the acetone ether phase while this same phase obtained from muscle was free of inhibition. Synthesis was not altered in tissue incubated in serum obtained from gilts at the 18th day of the cycle or the 24th day of gestation.

Exogenous progesterone can initiate regression of porcine corpora lutea at certain stages of the reproductive process (8). This induced regression is associated with a decreased luteal tissue weight of constant progesterone concentration (15), whereas naturally regressing corpora of late pregnancy have relatively constant fresh weight but a reduced progesterone concentration (5). Observed inhibition by late diestrual endometrial preparations is not believed to be associated with the progesterone content of the uterus since no progesterone could be detected (nor was any synthesized *in vitro*) in the volumes (4–8 ml.) of endometrial filtrate used in this study. Inclusion of up to 660 μg of progesterone in the Krebs bicarbonate medium per gram of luteal tissue did not inhibit synthesis. Estrogens (16) augment ovarian response to gonadotrophins in the rat. Under these *in vitro* conditions, however, addition of 1 mg. of sodium estrone sulfate, with or without 213 I.U. of lactogenic hormone, to Krebs bicarbonate medium did not alter progesterone synthesis. Incubation medium prepared from chick embryo extract (EE-50, 1–3 ml./10 ml. Krebs bicarbonate medium), furnishing high energy sources, did not alter *in vitro* progesterone synthesis.

TABLE 2. EFFECT OF SUBTOTAL HYSTERECTOMY ON PROGESTERONE CONCENTRATION IN LUTEAL TISSUE

Exp. No.	Day of cycle hysterectomy	Days to slaughter post-hysterectomy	Corpora lutea		Progesterone concentration			Av. luteal progesterone/animal
			No.	Wt.	Initial	After 2-hr. incubation	Increase	
				gm.	μg/gm.	μg/gm.	μg/gm.	μg
1*	7	25	4	2.1	73	115	42	153
2	7	25	12	7.2	97	146	49	698
3	7	50	16	5.5	58	104	46	319
4	15	50	17	6.0	70	115	45	420

* Animal showed adhesions and infection of ovaries and cervix at slaughter.

Bradbury *et al.* (9) stated that in some species apparently there are luteolytic substances, especially in the endometrium, which hasten involution of corpora lutea in non-fertile cycles. Inactivation or removal of this luteolysin may allow the corpus luteum to persist in an active state for a period of time equivalent to duration of pregnancy. Results of the investigation reported here suggest a relationship between life span of corpora lutea and endometrium. However, both the stimulatory and inhibitory activities observed with endometrial filtrates may be the result of non-specific substances present in endometrium. These substances may or may not be involved in the *in vivo* development, maintenance and regression of the corpus luteum during the estrous cycle.

A uterine effect on luteal activity may also be observed *in vivo*. Subtotal hysterectomy of gilts at 7 and 15 days after estrus caused maintenance of functional corpora lutea at least 50 days following surgery (Table 2) as indicated by initial progesterone content as well as the capacity of this luteal tissue to synthesize progesterone *in vitro*. Initial hormone concentrations 25 and 50 days post-hysterectomy corresponded to concentrations during the latter half of the progestational phase of the cycle and days 16 to 72 of gestation (5). Increases following incubation were similar to those of pregnant gilt luteal tissue. Luteal progesterone per animal was comparable to that present in animals during early gestation.

Data from this study indicate corpora lutea in gilts following hysterectomy are not only functional in terms of their capacity to synthesize progesterone *in vitro* but also increase in activity beyond their pre-hysterectomy status. Rowlands and Short (17) observed increased luteal size and progesterone concentration following hysterectomy in guinea pigs. These findings are in agreement with results of earlier observers who evaluated luteal function following hysterectomy in terms of duration of pseudopregnancy. Loeb (18) found duration of pseudopregnancy in hysterectomized guinea pigs was approximately 90 days as compared to 70 days for gestation. Asdell and Hammond (19) noted corpora lutea in mated hysterectomized rabbits retained their size and ovulation inhibiting function for 2 to 29 days, representing a life approximately 10 days longer than that

of the pseudopregnant corpus luteum, but about 6 days shorter than that of the corpus luteum of pregnancy. In the rat, Bradbury *et al.* (9) reported 12, 18 and 22 days for the length of pseudopregnancy, pseudopregnancy following hysterectomy and gestation, respectively. Complete removal of the uterus in the ewe and cow caused maintenance of the corpus luteum in most animals (20). Spies *et al.* (15) hysterectomized four gilts 7 days after the beginning of heat. None of the gilts had showed estrus by time of slaughter 32, 71, 72 and 119 days after the last estrus. According to Du Mesnil Du Buisson and Dauzier (21), corpora lutea persisted for 117 days and ovulation occurred about 200 days after the last estrus in hysterectomized pregnant and non-pregnant sows.

Hechter *et al.* (22) and Chu *et al.* (23) have demonstrated that effects of hysterectomy on persistence of corpora lutea could be abolished in the rat and rabbit by transplants of uterine tissue. However, a similar role for uterine transplants has not been observed in the guinea pig (18) or gilt (15). Mishell and Motyloff (24) reported bovine endometrial extract retarded ovarian atrophy following hysterectomy in the rabbit. Tenny *et al.* (14) reported similar findings following administration of an aqueous extract from a human uterus. Reynolds has reviewed other literature dealing with physiologic effects of hysterectomy in several species (25). The possibility of a uterine substance directly or indirectly influencing regression of corpora lutea in the non-fertile cycle of swine must be considered.

REFERENCES

1. Tepperman, J.: *Endocrinology* **47:** 384. 1950.
2. Saffran, M., B. Grad and J. Bayliss: *Endocrinology* **50:** 639. 1952.
3. Brady, R. O.: *J. Biol. Chem.* **193:** 145. 1951.
4. Hollander, N. and V. Hollander: *J. Biol. Chem.* **233:** 1097. 1958.
5. Duncan, G. W., A. M. Bowerman, W. R. Hearn and R. M. Melampy: *Proc. Soc. Exper. Biol. and Med.* **104:** 17. 1960.
6. Day, B. N., L. L. Anderson, L. N. Hazel and R. M. Melampy: *J. Animal Sci.* 18: 675. 1959.
7. Smith, V. R., W. H. McShan and L. E. Casida: *J. Dairy Sci.* **40:** 443. 1957.
8. Sammelwitz, P. H. and A. V. Nalbandov: *J. Animal Sci.* **17:** 1233. 1958.
9. Bradbury, J. T., W. E. Brown and L. A. Gray: *Recent Progress in Hormone Research* **5:** 151. 1950.
10. Hisaw, F. L.: *Yale J. Biol. Med.* **17:** 119. 1944.
11. Nalbandov, A. V.: Reproductive Physiology. W. H. Freeman and Co., p. 62. 1958.
12. Armstrong, D. T. and W. Hansel: *J. Dairy Sci.* **42:** 533. 1959.
13. Asdell, S. A.: *Physiol. Rev.* **8:** 313. 1928.
14. Tenny, B., F. Parker, Jr. and S. L. Robbins: *Am. J. Obst. Gyn.* **75:** 656. 1958.
15. Spies, H. G., D. R. Zimmerman, H. L. Self and L. E. Casida: *J. Animal Sci.* 19: 101. 1960.
16. Meyer, J. E. and J. T. Bradbury: *Endocrinology* **66:** 121. 1960.
17. Rowlands, I. W. and R. V. Short: *J. Endocrinol.* **19:** 81. 1959.
18. Loeb, L.: *Am. J. Physiol.* **83:** 202. 1927.
19. Asdell, S. A. and J. Hammond: *Am. J. Physiol.* **103:** 600. 1933.

20. Wiltbank, J. N. and L. E. Casida: *J. Animal Sci.* **15:** 134. 1956.
21. Du Mesnil Du Buisson, F. and L. Dauzier: Colloque Sur La Reproduction Et L'Insemination Artificelle Du Proc. Ann. Zootechnie, Series D. Supplement: 147. 1959.
22. Hechter, O., M. Fraenkel, M. Lev and S. Soskin: *Endocrinology* **26:** 680. 1940.
23. Chu, J. P., C. C. Lee and S. S. You: *J. Endocrinol.* **4:** 392. 1946.
24. Mishell, D. R. and L. Motyloff: *Endocrinology* **28:** 436. 1941.
25. Reynolds, S. R. M.: Physiology of the Uterus. P. B. Hoeber Inc., 2nd Ed., p. 503. 1949.

ALTERATIONS OF THE INTERSCAPULAR BROWN FAT (HIBERNATING GLAND) ADRENALS AND THYMUS DURING PREGNANCY IN HAMSTERS[1,2]

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ABSTRACT

Adrenals, thymus and the interscapular brown fat body (ISB) were examined in 60 pregnant and 60 virgin female hamsters of the same age and breed. Adrenals became significantly enlarged during pregnancy (25 mg./100 gm. vs 14 mg./100 gm. in the controls). Fasciculata cells were hypertrophic, granular and more lightly stained (H&E). Alkaline phosphatase activity was markedly increased in the adrenal cortex of pregnant as compared to control hamsters. The thymus diminished significantly in weight in pregnant hamsters (41 mg./100 gm. vs. 63 mg./100 gm. in the controls). Lymphocytolysis occurred in the subcapsular cortex. The ISB diminished progressively from 267 mg./100 gm. in nonpregnant hamsters to 78 mg./100 gm. during the last third of pregnancy. The number and size of intracellular fat vacuoles progressively decreased throughout pregnancy and the average cell diameter diminished from 23.3μ to 11.8μ. The number of signet ring cells in the ISB counted in 10 high power fields averaged 16 in the pregnant group and 55 in the controls. Considering that adrenal hypertrophy or cortisone treatment induces large accumulation of lipids in the ISB of normal hamsters, the unusual association of enlarged adrenals and depleted ISB in the pregnant hamster is discussed.

THE functional significance of brown adipose tissue is not clarified. Brown fat is found in many mammalian species including man (1). In laboratory animals its proportion in relation to white fat decreases progressively from birth to adulthood even in areas such as the interscapular space where brown fat is initially predominant (2). The interscapular brown fat body (ISB) is highly vascular. Its size and chemical composition are strongly influenced by hormones, stress and numerous nutritional and environmental conditions. It has the capacity of discharging its lipid content in a matter of hours in animals subjected to stress (1, 3) and is able to increase its weight three-fold by storing lipids in animals treated with cortisone (2). In conjunction with this activity, glycogen appears in the brown fat cells (4, 5). Metabolically brown fat is significantly more active

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[2] Part of the material contained in this paper was presented before the American Physiological Society, April, 1959, Atlantic City, N. J.

than white fat as shown by phosphorus turnover determinations and oxygen consumption measurements (6, 7, 8, 9).

In rodents, as well as in monkeys and apes, brown fat appears to be the main reservoir for the multiplication of all strains of poliomyelitis virus (10, 11). This discovery revived an interest in the physiology of brown fat, initiating a series of investigations concerned with the effect upon brown fat of various factors known to influence susceptibility to poliomyelitis. Pregnancy being a factor, its influence upon the interscapular brown fat is reported in this paper.

MATERIAL AND METHODS

Newborn female hamsters of the same breed were separated into two groups. When they reached the adult age and weight one of the grops was put in contact with males for three days for mating. The other group was kept as a control. The age of pregnancy was established as accurately as possible. Errors in estimation were retrospectively corrected at the time of delivery in all animals in which the ISB was surgically removed for study at various stages of the pregnancy. The average pregnancy in hamsters being of a duration of about 16 days it was divided for experimental convenience into three thirds of 5 days each. Groups of pregnant and virgin hamsters were periodically sacrificed and the adrenal glands, thymus and ISB were weighed and prepared for histologic and histochemical examination.

The alterations of the interscapular brown fat were studied also *in vivo* by surgical exposure and removal at various stages of the pregnancy. The precise delimitation of the brown fat body in the hamster renders the surgical removal simple and the estimation of various alterations fairly accurate. Histological examination followed the surgical removal of the ISB from both pregnant and control animals.

Histologic studies were performed on fragments of ISB, adrenals and thymus embedded in paraffin and cut at 5μ after fixation in 10% neutral formalin. The sections were stained with hematoxylin and eosin. Portions of the adrenal glands were also fixed in 80% alcohol. After embedding and cutting at 5μ they were stained for alkaline phosphatase activity following the procedure of Gomori (12). Differences between the averages of the weight of the glands in experimental and control groups were statistically analyzed using "Student's" formula.

RESULTS

To obtain the average weight of the adrenals, thymus and ISB in pregnant hamsters, figures from animals at all stages of pregnancy were computed together. The increase of adrenal mass as well as the decrease in weight of thymus and brown fat are statistically highly significant. (Table 1).

TABLE 1. ALTERATION OF THE RELATIVE WEIGHT (MG./100 GM. BODY WEIGHT) OF ADRENALS, THYMUS AND INTERSCAPULAR BROWN FAT IN PREGNANT AS COMPARED TO CONTROL HAMSTERS[1]

Group	No. of animals	Body wt., gm.	Adrenals, mg./100 gm.	P	Thymus, mg./100 gm.	P	ISB, mg./100 gm.	P
Pregnant	25	102	21 ± 5.2	0*	41 ± 12	0*	177 ± 33	0*
Control	40	104	14 ± 2.2		63 ± 9.9		235 ± 47	

[1] Deviations shown are standard deviation.
* 0.001.

TABLE 2. AVERAGE WEIGHT OF THE INTERSCAPULAR BROWN FAT (ISB) REMOVED SURGICALLY AT VARIOUS STAGES OF PREGNANCY IN HAMSTERS

Group	No. of animals	Body wt., gm.	ISB, wt., Mg.	P
Pregnant 1–5 days	19	85	163	
Pregnant 6–10 days	20	87	138	
Pregnant 11–15 days	13	86	78	
				0*
Virgin Control**	53	99	267	

* 0.001.

** Since no significant differences were found from a control group to another they were all pooled together in a common virgin control figure.

The adrenals of 35 pregnant and 35 virgin hamsters were examined histologically. The H & E stains showed enlargement of the cells of the outer and middle fasciculata and sometimes of the glomerulosa in pregnant hamsters. The cells appeared blown up, finely granular and more lightly stained. The number of nuclei was counted in 10 high power fields in the adrenals in 10 pregnant and 10 virgin control hamsters. The average count in pregnancy was 198 and in controls 241, suggesting that in pregnant animals the individual adrenal cells are hypertrophic. The cells were larger in pregnant than in nonpregnant animals (Fig. 1).

Forty specimens were fixed and stained for alkaline phosphatase activity. There was a marked increased in alkaline phosphatase activity in the adrenals of pregnant hamsters. It was present mainly in the outer and middle fasciculata and sometimes in the entire fasciculata, while control adrenals showed only a narrow zone of activity in the inner fasciculata and reticularis. The medulla and glomerulosa were inactive. (Fig. 2).

There was variation in enzyme activity. However, in general, a definite difference between pregnant and nonpregnant hamsters could be observed. Phosphatase activity was present in the cytoplasm, particularly near the cell border. Delicate fibers of the capsule and occasionally those of the sinusoids probably representing reticulum fibers, also showed enzyme activity. In active cells the nuclei were stained also.

The thymus of pregnant hamsters showed varying degrees of involution (Fig. 3). The figure shows reduction of lymphocytes in the cortex, particularly in areas under the capsule.

A progressive diminution in weight of the ISB occurred (Table 2). As compared to controls studied simultaneously the difference is highly significant.

Direct examination after surgical exposure revealed that with the progress of the pregnancy, the ISB became darker and in many instances hemorrhagic.

The ISB's of about 40 pregnant and 30 virgin female hamsters were examined histologically. The number and size of intracellular fat vacuoles decreased markedly and progressively throughout pregnancy, (Fig. 4) this

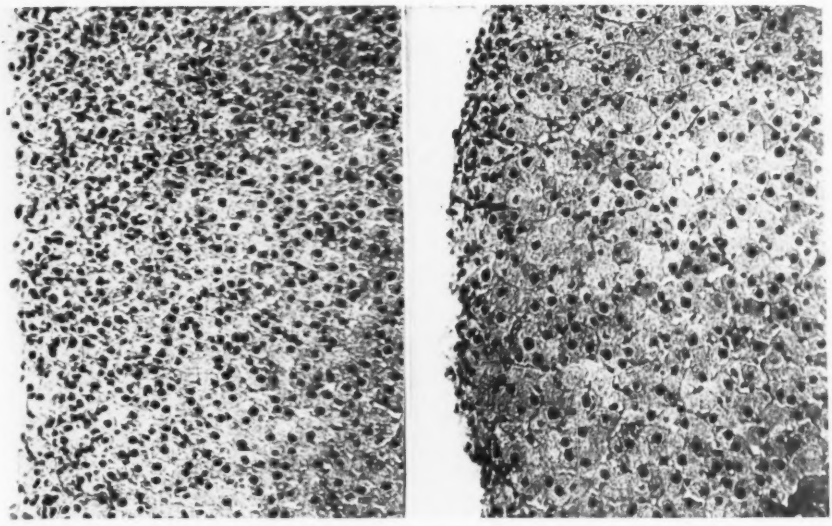

FIG. 1. Adrenal cortex of control at left and of pregnant hamster on right. The cortex of the pregnant hamster shows larger cells. (250×)

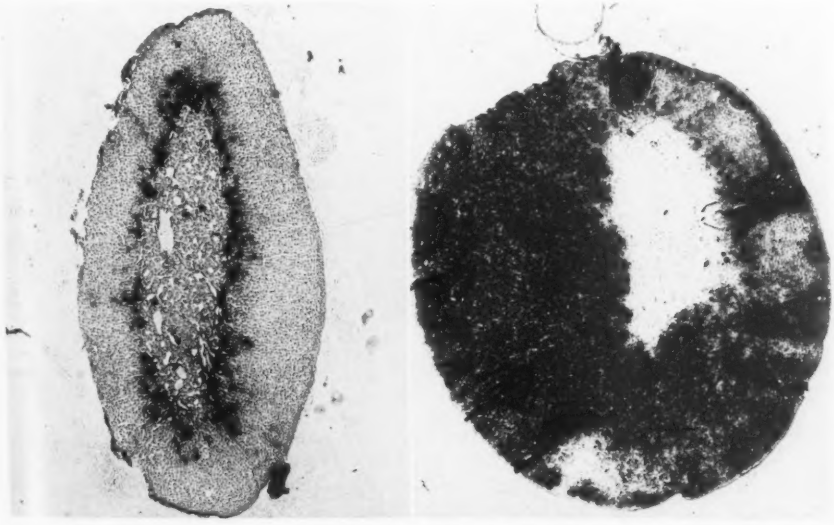

Fig. 2. Average alkaline phosphatase activity on adrenal sections of control at left and of pregnant hanster on right. The activity is confined to the reticularis and inner fasciculata in the former but extends over most of the cortex in the latter. (28×)

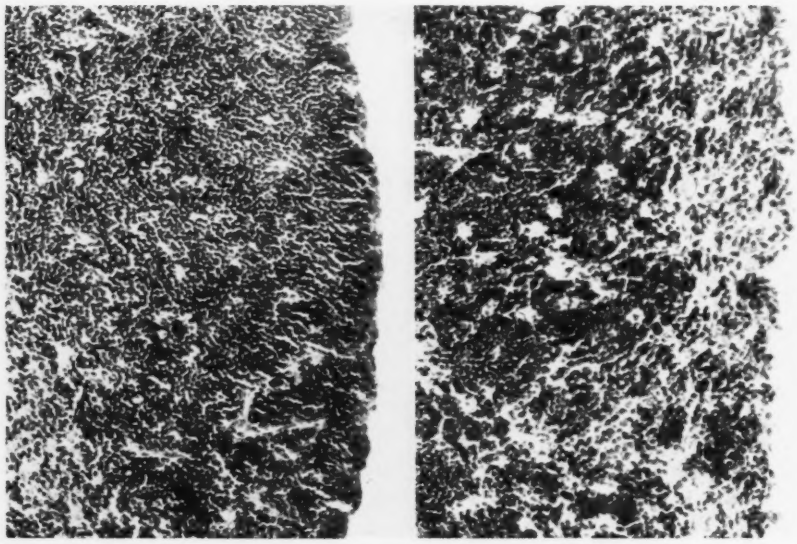

FIG. 3. Thymus sections of control at left, and of pregnant hamster on right. Reduction of lymphocytes in the subcapsular cortex is obvious in the latter as compared to the former. (107×)

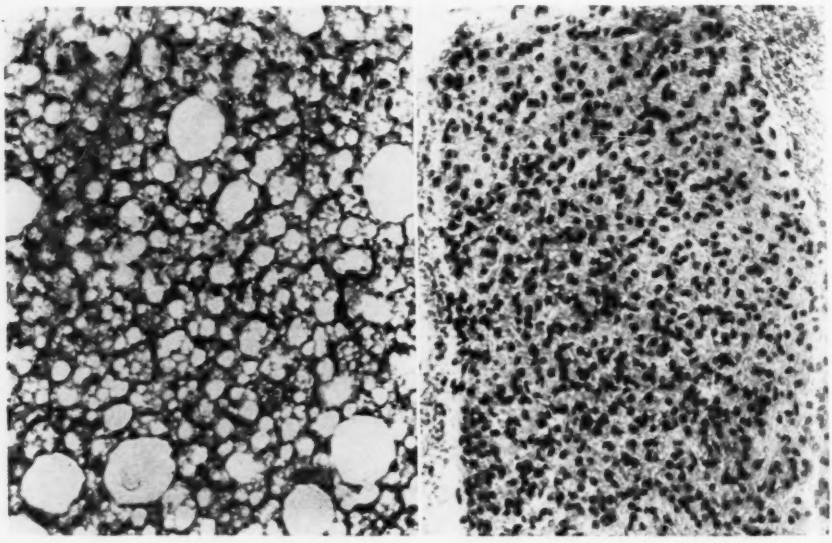

Fig. 4. Sections of the interscapular brown fat of control at left, and of pregnant hamster on right. Lipid depletion and absence of signet ring cells are characteristic in the latter as compared to the former. (250×)

TABLE 3. MEAN FAT CELL DIAMETER IN THE INTERSCAPULAR BROWN FAT OF PREGNANT AS COMPARED TO CONTROL HAMSTERS

Group	No. of animals	Cell diameter μ
Pregnant 1–10 days	10	17.3
Pregnant 11–15 days	5	11.8
Virgin Control	10	23.3

change corresponding roughly to the decrease in weight of the ISB. The cells became smaller (Table 3). The number of signet ring cells was markedly reduced in brown fat of pregnant animals. The average number counted in 10 high power fields was 16 in pregnant hamsters while in virgin controls it was 55.

DISCUSSION

From the facts presented above it is evident that in the pregnant hamster, the adrenal gland becomes enlarged while thymus and brown fat are significantly diminished in size. The histologic and histochemical studies of thymus and adrenal cortex suggest a hyperfunctional state of the latter. This is, however, not supported by the congested, lipid depleted brown fat. It has been shown previously that cortisone administration causes a dramatic increase while adrenalectomy is followed by significant decrease in size and lipid content of the brown fat (2, 6). Unpublished experiments in our laboratory demonstrate that among the hormones known to be prevalent during pregnancy, progesterone has significant lipid-depleting effects upon the brown fat of nonpregnant hamsters. A similar, although less significant, effect is exhibited by estrogens. Treatment with ACTH also provokes significant lipid depletion of the brown fat but only for the initial 48 hours. This effect is progressively reversed as adrenal hypertrophy develops under the influence of the treatment. When ACTH and cortisone are given together, the fat-depleting effect of the former is apparently overwhelmed by the stronger opposite effect of the latter. However, the lipid storing activity of combined treatment is inferior to that of cortisone alone indicating a certain degree of antagonism between the two hormones. These findings are consistent with reports of others (1, 3, 13) showing that under the influence of stress, brown fat becomes initially depleted then surcharged with lipids (1, 3), and that prolonged ACTH treatment which increases the lipid content of the ISB in the intact rat, decreases it to total depletion in the unilaterally adrenalectomized one (13).

It is not clear as yet whether the excess of lipid-depleting hormones in the pregnant hamster is solely responsible for the depletion of the brown fat, in spite of the hypertrophic adrenal cortex, or whether the hypertrophic adrenal cortex of the pregnant hamster is functionally different from that of the nonpregnant animal. The latter possibility is suggested by the altered alkaline phosphatase activity, which is confined to the zona reticularis in

the normal hamster but involves the entire cortex in the pregnant animal.

The disappearance of the signet ring cells concurrent to the progressive lipid depletion of the brown fat during pregnancy, strongly suggests that brown and white fat tissues represent morphologically reversible functional aspects of the same cellular element. This viewpoint also is supported by previous observations showing increased cell diameter, coalescence of lipid vacuoles and peripheral migration of nuclei of ISB in the cortisone treated hamster (2).

REFERENCES

1. Timiras, P. S.: *Annales de l'Acfas* **16**: 124. 1950.
2. Aronson, S. M., Teodoru, C. V., Adler, M. and Shwartzman, G.: *Proc. Soc. Exp. Biol. & Med.* **85**: 214. 1954.
3. Lemonde, P. and Timiras, P. S.: *Revue Canadienne de Biologie* **10**: 76. 1951.
4. Fawcett, D. W.: *Endocrinology* **42**: 454. 1948.
5. Engel, F. L., Fredericks, J., Lopez, E. and Albertson, T.: *Endocrinology* **63**: 768. 1958.
6. Fawcett, D. W. and Jones, I. C.: *Endocrinology* **45**: 609. 1949.
7. Littrell, J. D., Martin, D. and Hartman, C. G.: *Anat. Rec.* **89**: 39 (Abstract) 1944.
8. Mirski, A.: *Biochem. J.* **36**: 232. 1942.
9. Hook, W. E. and Barron, E. S. G.: *Am. J. Physiol.* **133**: 56. 1941.
10. Aronson, S. M., and Shwartzman, G.: *Am. J. Path.* XXIX: 381. 1953.
11. Shwartzman, G., Aronson, S. M., Teodoru, C. V., Adler, M., and Jahiel, R.: *Ann. N. Y. Acad. Sci.* **61**: 869. 1955.
12. Gomori, G.: *Proc. Soc. Exper. Biol. & Med.* **42**: 23. 1939.
13. Bole, G. G., Jr., B. L. Baker, Ingle, D. J., and Li, C. H.: *Univ. of Michigan Med. Bull.* **27**: 413. 1951.

PHARMACOLOGICAL CHARACTERISTICS OF THE ACTIVE PRINCIPLES IN NEUROHYPOPHYSIAL EXTRACTS FROM SEVERAL SPECIES OF FISHES[1]

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ABSTRACT

Pituitary extracts from a marine teleost (*Pollachius virens*), a cyclostome (*Petromyzon marinus*) and an elasmobranch (*Squalus acanthias*) have been subjected to a variety of biological assays. The activities of these extracts have been determined on rat blood pressure, rat antidiuresis, rat uterus *in vitro*, rabbit milk ejection, fowl blood pressure, the hen oviduct *in vitro* and the bullfrog bladder *in vitro*. The patterns of relative activities on these assays for each species have been compared to the patterns for known neurohypophysial hormones and related synthetic analogues. The evidence suggests that arginine vasotocin (8-arginine oxytocin) is the major active neurohypophysial peptide in the teleost and cyclostome. Oxytocin also appears to be present in the teleost. The neurointermediate lobe of the elasmobranch contains a pattern of activities that cannot be readily attributed to any of the known neurohypophysial peptides or synthetic analogues that have been examined to date.

MUNSICK *et al.* (1, 2) demonstrated that the chicken neurohypophysis contains at least two active peptides. One appears to be oxytocin. The second peptide has pharmacological and chromatographic characteristics indistinguishable from those of arginine vasotocin (8-arginine oxytocin). Acher *et al.* (3) have subsequently isolated a peptide from chicken neural lobes that contains the same amino acids as mammalian oxytocin. Chauvet *et al.* (4) also found a peptide containing the amino acids of arginine vasotocin in chicken extracts as well as a third peptide resembling arginine vasopressin.

Arginine vasotocin was synthesized by Katsoyannis and du Vigneaud (5) who generously supplied it to this Department for pharmacological studies. It contains the ring structure of oxytocin and the side chain of arginine vasopressin. Its pharmacological properties are unique among the natural and synthetic peptides that we have studied in that it is highly active in causing contraction of the hen oviduct (2, 6). It is also extremely active in increasing water uptake by frogs (the "frog water balance re-

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sponse"), in increasing water permeability of the frog bladder (7), in inhibiting diuresis in frogs (8) and in promoting sodium transport by isolated frog skin (9).

Heller (10) originally observed that cod pituitary extract contains frog water balance activity far out of proportion to its mammalian oxytocic or antidiuretic activities. More recently Pickering and Heller (11) have separated the "water balance principle" in teleost pituitary extracts from oxytocin by paper chromatography. They also found that a partially purified sample of this fraction, when hydrolyzed, yielded the eight amino acids of arginine vasotocin, as well as alanine and an unidentified peptide fragment (12). Our pharmacological investigations have also led us to suggest that the frog water balance principle and arginine vasotocin are identical (7, 13). It therefore appears likely that arginine vasotocin is indeed the water balance principle in teleost pituitary extracts (12, 13, 14). Its apparent presence in fowl and teleost pituitaries and its suggested presence in other vertebrates (13) makes a pharmacological investigation of its distribution among the fishes of considerable interest.

In this paper we report a detailed examination of the pharmacological properties of pituitary extracts from representatives of three major classes of fishes: a teleost, an elasmobranch and a cyclostome. We also compare the activities found to those of neurohypophysial hormones and their synthetic analogues in an attempt to arrive at a tentative identification of the active principles in the neurohypophyses of these fishes. Preliminary notes covering parts of this investigation have appeared elsewhere (7, 13, 15, 16).

MATERIALS AND METHODS

Two preparations from pollack (*Pollachius virens*) pituitaries have been studied. One was a lyophilized powder of whole pollack pituitaries (designated Fp 504 by Wilhelmi). The other was a partially purified preparation identified by Wilhelmi *et al.* (17) as fraction F6SF-64B. This contained approximately a 10% admixture of hake and cod pituitaries but was obtained predominantly from pollack. The details of its preparation are given by Wilhelmi *et al.* (17). The pituitaries for both preparations were collected by Dr. Grace E. Pickford of the Bingham Oceanographic Laboratory of Yale University. This collection was part of an extensive program supported by continuing grants to Dr. Pickford from the National Science Foundation. Both pollack powders were prepared by Dr. Alfred E. Wilhelmi of the Department of Biochemistry, Emory University. In the tables these preparations are identified as "whole" and "fraction" respectively.

Lamprey (*Petromyzon marinus*) material was collected with the assistance of Dr. Willard D. Roth of the Department of Anatomy, Harvard Medical School. Migrating lampreys were captured by hand in the Exeter (N.H.) River. They were immediately decapitated and the portion of the ventral forebrain containing the neurohypophysis was removed by block dissection and dropped into acetone. The inclusion of much extrapituitary tissue was unavoidable and this probably accounts in part for the low specific activity of the final powder.

Two preparations of spiny dogfish (*Squalus acanthias*) neurointermediate lobes were also examined. One was derived from pituitaries of mature male dogfish and generously

sent to us by Drs. A. M. Perks, M. H. I. Dodd and J. M. Dodd of the Gatty Marine Laboratory, University of St. Andrews, Scotland. The other was prepared from neurointermediate lobes from adults of both sexes collected by one of us (R.A.M.) in the Gulf of Maine. Both were acetone-dried powders and are identified in Table 2 as "Scotland" and "Maine" powders respectively.

Dried pituitary preparations were homogenized when necessary and extracted in 0.3% acetic acid, 0.5% chlorobutanol and 0.9% NaCl in a boiling water bath for 5 minutes. After centrifugation the supernatant was sealed in ampules and kept under refrigeration until used for assays.

Rat vasopressor assays were performed by the method of U.S.P. XV. Intravenous rat antidiuretic assays have been described elsewhere (2, 18). Rat uterus assays were performed *in vitro* in solutions containing either no magnesium or 0.5 mM/1 of this cation according to Munsick's (19) modification of the Holton (20) technique. Rabbit milk ejection assays were performed essentially as described by van Dyke *et al.* (21). Fowl vasodepressor assays were similar to those described in U.S.P. XV except that unanesthetized hens were used and a 2×2 design was followed in administering doses of standard and unknown (2). Fowl oviduct *in vitro* assays are described by Munsick *et al.* (2). Frog bladder assays were performed *in vitro* as described elsewhere (7). U.S.P. reference standard posterior pituitary powder was used in all assays except on the frog bladder. Synthetic oxytocin (Syntocinon) solution that had been carefully restandardized against the U.S.P. standard was used for a working standard in this assay.

Neurohypophysial hormones and their analogues were similarly assayed. Dr. V. du Vigneaud and his coworkers of the Department of Biochemistry, Cornell Medical College, generously supplied us with samples of highly purified natural arginine vasopressin, lysine vasopressin and oxytocin as well as purified synthetic arginine vasotocin (8-arginine oxytocin), oxypressin (3-phenylalanine oxytocin or 8-leucine vasopressin) and 8-histidine vasopressin. The late Dr. D. A. McGinty of Parke, Davis and Co., Detroit, supplied us with a sample of highly active natural arginine vasopressin (Pitressin of bovine origin) powder. Drs. B. Berde and A. Cerletti of Sandoz, Ltd., Basle, and Dr. R. Bircher of Sandoz pharmaceuticals, Hanover, N. J., kindly made available solutions of synthetic oxytocin (Syntocinon), 8-lysine oxytocin, 3-valine oxytocin, 2-phenylalanine oxytocin, 8-leucine vasopressin, 8-lysine vasopressin and 2-phenylalanine, 8-lysine vasopressin. These preparations are indicated in Table 3 as being "in solution."

Values for the assays indicated in the tables are results of single assays or are weighted means of the estimates from several assays of the same preparation (2). Figures in parentheses represent 95% confidence limits of the estimates when these limits could be conveniently calculated. If confidence limits are not indicated the figures represent geometric means of the estimates of activity on several assays (2).

Standard errors for some activity ratios are also indicated in the tables. The standard error of a ratio has been calculated on the assumption that the standard error of the logarithm of the potency ratio is equivalent to the square root of the sum of the squares of the standard errors of the logarithms of the potency estimates that enter into the ratio. In assessing the "significance" of differences between such ratios a P value has been assumed based on a value of t corresponding to the number of degrees of freedom in the individual assay contributing to the ratio that entails the fewest degrees of freedom. This would introduce bias tending to make the estimate of P greater, if anything, than its true value, thus providing a conservative basis for judging significance. P thus calculated is considered to indicate significance only when less than 0.01. The authors are indebted to Dr. John W. Fertig of the Department of Biostatistics, Columbia University School of Public Health and Administrative Medicine, for helpful suggestions concerning the treatment of these data.

TABLE 1. THE ACTIVITIES OF FISH PITUITARY EXTRACTS COMPARED TO THOSE OF ARGININE VASOTOCIN ON A VARIETY OF ASSAYS

Assays	Activities (95% confidence limits in parentheses)			
	Arginine vasotocin U./mg.	Pollack pituitary preparations		Lamprey pituitary mU./mg.
		Whole U./mg.	Fraction U./mg.	
Rat vasopressor	64 (62–67)	0.44 (0.38–0.50)	5.5 (5.1–5.9)	7.7 (7.3–8.1)
Rat antidiuresis	74 (44–120)		5.4	10
Rat uterus without Mg	37 (34–41)	0.84 (0.76–0.92)	9.6 (9.0–10.5)	5.3 (5.1–5.4)
Rat uterus with Mg 0.5 mM	71 (65–78)			10.1 (7.2–14.1)
Rabbit milk ejection	79 (75–82)		20.3 (15–28)	10.3 (9.4–11.2)
Hen oviduct	640	4.8 (4.1–5.5)	25.2 (18–35)	65 (54–76)
Frog bladder	10,000 (8700–11,600)	109 (72–205)	1360 (965–1920)	1020 (440–2400)
	Activity ratios (± standard error of the ratios)			
$\frac{\text{Rat uterus without Mg}}{\text{Rat vasopressor}}$	0.59 ± 0.08	1.91 ± 0.15	1.75 ± 0.08	0.69 ± 0.05
$\frac{\text{Frog bladder}}{\text{Rat vasopressor}}$	160 ± 20	250 ± 31	247 ± 31	133 ± 37

RESULTS

Pollack. The activities of extracts of whole pollack pituitaries and of the partially purified fraction on selected assays are shown in Table 1. The activities of the fish extracts on the frog bladder and hen oviduct are extremely high relative to their activities on rat blood pressure or rat uterus assays. Arginine vasotocin is the only known peptide that has similarly high relative activities on both hen oviduct and frog bladder (Table 1). The ratios of frog bladder to rat vasopressor activities, given at the bottom of Table 1, for the pollack pituitary extracts are not significantly different from this ratio for arginine vasotocin.

The pollack extracts are more potent when assayed on the rat uterus and on rabbit milk ejection than they would be if they contained only arginine vasotocin. The ratios of rat uterus to rat vasopressor activity (Table 1) for the pollack extracts are significantly greater than is this ratio for arginine vasotocin. This we believe to be due to the presence of some oxytocin in these pituitary extracts. This is consistent with the finding of Pickering and Heller (11) of two active peptides in pollack pituitary extracts. They believe that one is oxytocin. The other contains most of the frog water balance activity and may well be, therefore, arginine vasotocin (12).

Lamprey. Oxytocic (22, 23), water balance (22), vasopressor and antidiuretic (23) activities have been previously demonstrated in extracts of the lamprey forebrain. The lamprey extract examined in the current study also possesses activities typical of active neurohypophysial principles (Table 1).

The extract we have studied contained other structures of the ventral forebrain as well as the neurohypophysis. Such structures may well contribute to the total active peptides extracted. For simplicity, however, we hereafter refer to this extract as "pituitary." The activities of the lamprey extract on the assays shown are completely consistent with those of arg-

TABLE 2. THE ACTIVITIES OF DOGFISH (*Squalus*) NEUROINTERMEDIATE LOBE EXTRACTS COMPARED TO THOSE OF ARGININE VASOTOCIN ON A VARIETY OF ASSAYS

Assays	Activities (95% Confidence Limits shown in parentheses)		
	Arginine vasotocin U./mg.	Squalus neurointermediate lobe extracts	
		Scotland powder mU./mg.	Maine powder mU./mg.
Rat vasopressor	64 (62–67)		c. 1.4
Rat antidiuresis	74 (44–120)	0.8	0.9 (0.4–2.0)
Rat uterus without Mg	37 (34–41)	35 (32–39)	30 (26–34)
Rat uterus with Mg 0.5 mM	71 (65–78)	71 (59–85)	38 (31–50)
Rabbit milk ejection	79 (75–82)	64 (59–70)	56 (49–63)
Avian depressor	110 (82–150)	c. 62	100 (95–107)
Hen oviduct	640	c. 5	25 (22–27)
Frog bladder	10,000 (8700–11,600)	930	623 (483–805)
	Activity ratios (±standard error of the ratios)		
Rat uterus without Mg. / Rat antidiuresis	0.50 ± 0.07	44	32 ± 10
Frog bladder / Rat antidiuresis	137 ± 19	1160	670 ± 270

nine vasotocin. In this case there is little evidence for the presence of oxytocin since ratios of rat uterus to vasopressor activities of the lamprey powder and of arginine vasotocin are not significantly different.

Dogfish. The distribution of activities of the two *Squalus* powders is shown in Table 2. Rat vasopressor activity is very weak and partially obscured by a vasodepressor effect. The vasopressor action appears consistent, however, if large doses are administered. It can be eliminated by pretreatment of the extract with 0.01 M sodium thioglycolate for two hours at room temperature and pH 7.4 (Fig. 1). The vasodepressor effect persists

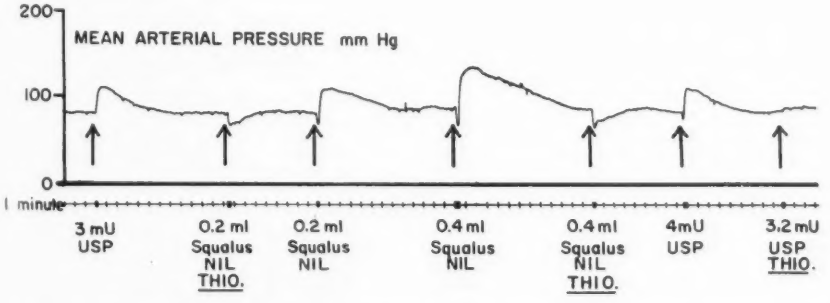

FIG. 1. Portion of an assay of the Maine *Squalus* neurointermediate lobe extract on the blood pressure of a rat. The extract (*Squalus* NIL) contained the material extracted from 10 mg of dry neurointermediate lobes/ml. U.S.P. Posterior Pituitary Reference Standard (U.S.P.) was used as a standard. The preparations marked "THIO" had been incubated for two hours at room temperature in 0.01 M thioglycolate, at pH 7.4.

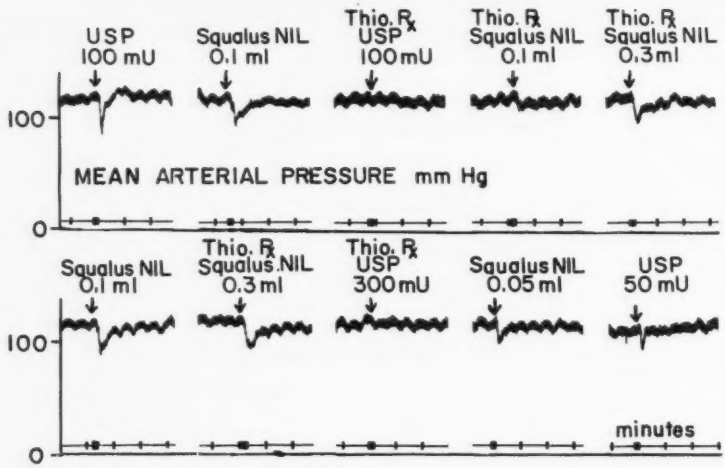

FIG. 2. Portion of an assay of the Maine *Squalus* neurointermediate lobe extract (*Squalus* NIL) on the blood pressure of the fowl. Each ml. of extract represents 10 mg. of dry neurointermediate lobes. The preparations marked "Thio Rx" had been incubated with 0.01 M thioglycolate for 2 hours at room temperature and pH 7.4. U.S.P. Posterior Pituitary Reference Standard (U.S.P.) was used as standard.

after thioglycolate treatment and cannot be considered to be an action of a typical neurohypophysial peptide. Doses of extract equivalent to 3 and 4 mg. of dry neurointermediate lobe were injected in order to obtain clear vasopressor responses. It is not surprising that extracts of this large amount of tissue contain thioglycolate-stable vasodepressor agents.

Perks *et al.* (24) failed to demonstrate vasopressor activity in elasmobranch extracts. They probably did not, however, use such large doses. They clearly demonstrated the presence of antidiuretic activity that was destroyed by thioglycolate. Our figures on rat antidiuretic activity agree quite well with theirs. Since the vasopressor potency of our *Squalus* extract could not be estimated accurately in the presence of vasodepressor activity we have chosen to use our figures for antidiuretic activity in calculating the activity ratios shown in Table 2. Although intravenous antidiuretic assays are less precise than vasopressor assays they are much more sensitive and therefore require the injection of much less tissue extract. Consequently they are less prone to interference from nonspecific components of the extracts.

Perks *et al.* (24) did not report fowl vasodepressor assays on their elasmobranch neurointermediate lobe extracts. The determination of the fowl vasodepressor action of the *Squalus* extracts was also complicated by the presence of a vasodepressor component that was not destroyed by thioglycolate (Fig. 2). The doses used were large, however, representing extracts of

0.5 to 1.0 mg. of dry neurointermediate lobe. The assays yield values that are undoubtedly high due to the inclusion of a vasodepressor contaminant. That approximately ¾ of the fowl vasodepressor activity is destroyed by thioglycolate, however, lends some confidence to the belief that a major portion of this activity is due to a neurohypophysial peptide containing a disulfide linkage similar to that of oxytocin and other known active neurohypophysial peptides.

Our antidiuretic, oxytocic and milk ejection assays on both *Squalus* extracts agree very well with those reported by Perks *et al.* (24). We report lower milk ejection activity relative to rat uterus activity. This difference is in large part due to differences in definitions of a milk ejection unit. Perks *et al.* (24) used oxytocin (Pitocin) as standard and considered a milk ejection unit to be the milk ejection activity of one oxytocic (rat uterus) unit of oxytocin. We use U.S.P. standard and consider one milk ejection unit to be the activity of one unit (0.5 mg.) of the standard ox posterior lobe powder. One unit of oxytocin (standardized by rat uterus (BP) or fowl vasodepressor (BP or U.S.P.) assays) has only 75–80% of the milk ejection activity of one unit (0.5 mg.) of the U.S.P. standard which contains both arginine vasopressin and oxytocin. Since our milk ejection unit is larger than that of Perks *et al.* (24) our assay results appear correspondingly lower. If allowance is made for this our milk ejection assay results are in good agreement with theirs.

DISCUSSION

The function of the neurohypophysis in all classes of fishes remains obscure (25). There is little evidence indicating that neurohypophysial hormones are concerned with osmoregulation in fishes although they appear to be concerned with the physiological regulation of water metabolism in all terrestrial tetrapods (16). There are anatomical grounds for the hypothesis that the neurohypophysis in fishes functions primarily as an organ for the secretion of hormones that have a local action in the modulation of adenohypophysial functions (16, 26, 27, 28). There is also evidence that neurohypophysial peptides may also be concerned with the initiation of spawning behavior in some teleosts (17, 29). It is evident, however, that fish pituitaries contain respectable quantities of active principles that appear analogous to the octapeptides that have been isolated and identified in mammalian neurohypophysial extracts.

The application of the multiple assay technique to fish pituitary extracts can provide pharmacological evidence that may offer valuable information as to what the active peptides can be, and more certainly, what they cannot be. Such an investigation is handicapped by the lack of suitable assay techniques utilizing species closely related to those providing the extracts. The only published assay technique using teleosts is the spawning

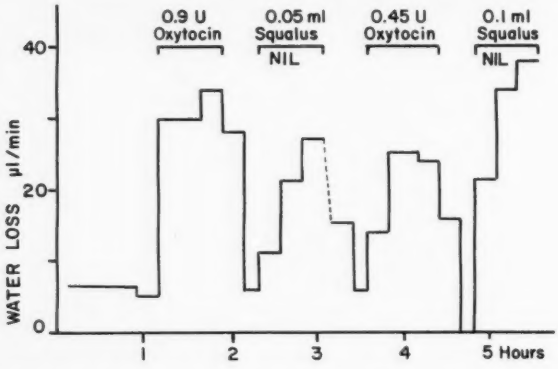

FIG. 3. A four point assay on the isolated bullfrog bladder of *Squalus* neurointermediate lobe extract (*Squalus* NIL) compared to oxytocin. One ml. of the extract represents 10 mg. of dry neurointermediate lobes.

reflex assay of Pickford (17). It is unfortunately of a very low order of sensitivity and also shows little discrimination between neurohypophysial peptides.

The *Squalus* neurointermediate lobe extracts show maximum activity on the frog bladder assay (Table 2, Fig. 3). In this respect they resemble arginine vasotocin (8-arginine oxytocin) and its lysine analogue (8-lysine oxytocin) (Table 3). Both these analogues, however, contain too much

TABLE 3. COMPARISON OF FROG BLADDER ACTIVITY OF *Squalus* NEUROINTERMEDIATE LOBE EXTRACT RELATIVE TO RAT VASOPRESSOR AND RAT UTERUS ACTIVITY TO THESE ACTIVITIES OF NATURAL AND SYNTHETIC ANALOGUES OF NEUROHYPOPHYSIAL PEPTIDES

Preparation	Activity ratios		Highest measured activity	
	Frog bladder Vasopressor	Frog bladder rat uterus	Assay	Units/mg.
Squalus neurointermediate lobe extract	445	21	Frog bladder	0.62
Arginine vasotocin (8-arginine oxytocin)	160	270	Frog bladder	10,000
8-lysine oxytocin	10	15	Frog bladder	in solution
Oxytocin*	52	0.9	Rat uterus	420
3-valine oxytocin	—	0.2	Milk ejection	in solution
2-phenylalanine oxytocin	—	0.6	Milk ejection	82
Arginine vasopressin*	0.23	4.8	Vasopressor	300
Lysine vasopressin*	<0.025	<1	Vasopressor	200
Oxypressin (8-leucine vasopressin)	<7	<1.2	Milk ejection	88
2-phenylalanine, 8-lysine vasopressin	<0.08	—	Vasopressor	in solution
8-histidine vasopressin	<6	<1	Milk ejection	18

* Highly purified natural neurohypophysial peptides. Higher absolute potencies have been observed with other preparations of these peptides.

vasopressor and antidiuretic activity to account for the frog bladder activity of the *Squalus* extracts (Table 3). In addition Perks *et al.* (24) report that both the antidiuretic and oxytocic activities of elasmobranch extracts resist tryptic digestion. This indicates that the active principle cannot contain an 8-arginine or 8-lysine residue. Peptides with such amino acid moieties are destroyed by trypsin. Tryptic digestion of arginine vasotocin has been observed by Munsick *et al.* (2).

The relatively high milk ejection, rat uterus, and fowl vasodepressor activities of the *Squalus* neurointermediate lobe extracts resemble those of oxytocin and its 2-phenylalanine, 3-phenylalanine (oxypressin), and 3-valine analogues. None of these has relative frog bladder activity comparable to the *Squalus* extract, however, and they can thus be excluded as possibly being responsible for the frog bladder activity of the *Squalus* extract (Table 3).

From our studies on structure-activity relationships of the available analogues of neurohypophysial hormones it should be possible to make some tentative suggestions as to what molecular configuration might possess the activities found in the *Squalus* extracts. Resistance to tryptic digestion (24) and the weak vasopressor and antidiuretic activities of the *Squalus* extracts make the presence of 8-arginine or 8-lysine unlikely. The ring of oxytocin is characteristic of analogues that have high frog bladder activity (oxytocin, arginine vasotocin, 8-lysine oxytocin) (Table 3). It would seem reasonable to suggest a search in the elasmobranch neurointermediate lobe for analogues containing the oxytocin ring and a substitution in the 8-position other than arginine, lysine or leucine. Unfortunately no such synthetic analogues are yet available. This is, of course, merely a guess but it may be of value as a guide to the eventual isolation of the active elasmobranch principle and in the elucidation of its amino acid sequence once its constituent amino acids have been identified.

If we assume that the few species studied are representative of the classes from which they came it should be possible to formulate a tentative scheme for the evolution of neurohypophysial principles in the fishes. Such an assumption is supported by the observation of Pickering and Heller (11) that two other teleosts (trout and cod) possess active principles closely resembling those of the pollack and the recent report by Maetz *et al.* (30) on the pharmacological similarity of extracts from four teleosts (mackerel, albacore, blenny and trout) when assayed for the ability to promote sodium transport by the isolated frog skin. Preliminary investigation of carp and hake pituitary extracts indicate that they are quite similar to pollack extracts. Perks *et al.* (24) have also reported that rays as well as dogfish of another genus (*Scyliorhinus*) possess neurohypophysial activities that resemble closely those found in *Squalus*.

Peptides resembling those of the vertebrate neurohypophysis have not

been convincingly demonstrated in invertebrates thought to be closely related to the ancestors of the vertebrates (31). The cyclostomes are the most primitive living vertebrates. A member of this group, the marine lamprey, appears to possess arginine vasotocin. We can suggest, therefore, that this is the most primitive known neurohypophysial peptide. The bony fishes appear to have inherited this principle from the cyclostomes and arginine vasotocin also appears to be present in all tetrapods except the mammals (13, 16). In mammals arginine or lysine vasopressin displaces arginine vasotocin as the vasopressor-antidiuretic principle.

Oxytocin appears in the bony fishes. It survives, apparently unchanged, throughout the subsequent evolution of the tetrapods. Its only known physiological functions, however, are directly related to mammalian reproduction and lactation. Its physiological significance in nonmammalian vertebrates remains unknown.

The elasmobranchs appear to have evolved along different lines than the other jawed vertebrates and have a unique neurohypophysial peptide (or peptides). We are not yet in a position to suggest its identity with any degree of confidence.

REFERENCES

1. Munsick, R. A., W. H. Sawyer and H. B. van Dyke: *Fed. Proc.* **18:** 110. 1959.
2. Munsick, R. A., W. H. Sawyer and H. B. van Dyke: *Endocrinology* **66:** 860. 1960.
3. Acher, R., J. Chauvet and M.-T. Lenci: *Biochim. Biophys. Acta* **38:** 344. 1960.
4. Chauvet, J., M.-T. Lenci and R. Acher: *Biochim. Biophys. Acta* **38:** 571. 1960.
5. Katsoyannis, P. G. and V. du Vigneaud: *J. Biol. Chem.* **233:** 1352. 1958.
6. van Dyke, H. B., R. A. Munsick and W. H. Sawyer: *Fed. Proc.* **18:** 163. 1959.
7. Sawyer, W. H.: *Endocrinology* **66:** 112. 1960.
8. Uranga, J. and W. H. Sawyer: *Am. J. Physiol.* **198:** 1287. 1960.
9. Sawyer, W. H.: *Nature*, **187:** 1030. 1960.
10. Heller, H.: *J. Physiol.* **100:** 124. 1941.
11. Pickering, B. T. and H. Heller: *Nature* **184:** 1463. 1959.
12. Heller, H. and B. T. Pickering: *J. Physiol.* **153:** 56P. 1960.
13. Sawyer, W. H., R. A. Munsick and H. B. van Dyke: *Nature* **184:** 1464. 1959.
14. Katsoyannis, P. G. and V. du Vigneaud: *Nature* **184:** 1465. 1959.
15. Sawyer, W. H., R. A. Munsick and H. B. van Dyke: *Fed. Proc.* **19:** 167. 1960.
16. Sawyer, W. H., R. A. Munsick and H. B. van Dyke: *Circulation* **21:** 1027. 1960.
17. Wilhelmi, A. E., G. E. Pickford and W. H. Sawyer: *Endocrinology* **57:** 243. 1955
18. Sawyer, W. H.: *Endocrinology* **63:** 694. 1958.
19. Munsick, R. A.: *Endocrinology* **66:** 451. 1960.
20. Holton, P.: *Brit. J. Pharmacol.* **3:** 328. 1948.
21. van Dyke, H. B., K. Adamsons, Jr. and S. L. Engel: *Rec. Prog. in Hormone Research* **11:** 1. 1955.
22. Lanzing, W. J. R.: *Acta Endocrinol.* **16:** 277. 1954.
23. Sawyer, W. H.: *Fed. Proc.* **14:** 130. 1955.
24. Perks, A. M., M. H. I. Dodd and J. M. Dodd: *Nature* **185:** 850. 1960.
25. Pickford, G. E.: in Gorbman (ed) *Comparative Endocrinology*. New York, John Wiley & Sons. 1959.

26. Green, J. D.: *Amer. J. Anat.* **88:** 225. 1951.
27. Green, J. D. and D. S. Maxwell: in Gorbman (ed) *Comparative Endocrinology.* New York, John Wiley & Sons. 1959.
28. Jørgensen, C. B. and L. O. Larsen: *Ergebn. der Biol.* **22:** 1. 1960.
29. Egami, N.: *Annot. Zool. Japon.* **32:** 13. 1959.
30. Maetz, J., F. Morel and B. Lahlouh: *Nature* **184:** 1236. 1959.
31. Sawyer, W. H.: *Endocrinology* **65:** 520. 1959.

CORTICOSTEROID SECRETION FOLLOWING CAVAL CONSTRICTION IN DOGS WITH ISOLATED PITUITARIES[1]

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ABSTRACT

The output per minute of 17-hydroxycorticosteroids was measured before and after partial thoracic inferior vena caval constriction in normal dogs and those with brain removal and isolated pituitaries. The "resting" control 17-hydroxycorticosteroid output was higher in dogs with brain removal than in normal animals. Both groups of dogs demonstrated a significant increase in corticosteroid output following caval constriction. It is concluded that partial constriction of the thoracic inferior vena cava is a strong stimulus to ACTH release.

DOGS with total brain removal down to the pons, but with intact pituitaries, demonstrate elevated basal outputs of corticosteroids from the adrenal (1). In addition, a significant number of these animals respond to peripheral nerve stimulation with a further increase in corticosteroid output. Other usually effective adrenal cortical stimulating procedures have been carried out in dogs with isolated pituitaries to determine the reactivity of these animals. One of the most effective has been partial constriction of the thoracic inferior vena cava. This report describes the effect of vena caval constriction on adrenal cortical secretion in normal dogs and in those with isolated pituitaries.

MATERIAL AND METHODS

Adult male mongrel dogs ranging in weight from 10 to 15 kg. were used in these experiments. Adrenal vein cannulations were carried out according to the technique of Hume and Nelson (2). 17-Hydroxycorticosteroids were measured by the method of Nelson and Samuels (3). At the time of adrenal vein cannulation a separate thoracic incision was made and the phrenic nerve was carefully dissected from the thoracic inferior vena cava. An infant endotracheal tube balloon was passed around the inferior vena cava just above the diaphragm and the two ends were tied together. It is important to apply the deflated balloon loosely to avoid compromising the venous lumen. Caval constriction was produced by introducing air into the balloon through a small rubber tube which was brought through a stab wound to the outside of the chest.

On the day following adrenal vein cannulation and thoracic caval cuffing, the animals were anesthetized with 30 mg./kg. of Nembutal, intravenously. Following anesthetiza-

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tion, plastic cannulas were placed in the femoral artery and inferior vena cava, and were connected to an arterial mercury manometer and venous water manometer, respectively. Rectal temperatures were monitored with indwelling telethermometers. Before and after arterial cannulation, intermittent samples of adrenal venous blood were collected and analyzed for 17-hydroxycorticosteroids.

Four hours after blood vessel cannulation adrenal venous samples were obtained and partial thoracic vena caval constriction was obtained by inflating the balloon. Venous pressure elevation of 10 cm. was achieved in the inferior vena cava below the constriction. The elevation in venous pressure remained quite constant throughout the experiment and was maintained for 45 minutes, at which time the balloon was deflated and the venous pressure rapidly fell to normal. Adrenal venous blood samples were obtained before and after caval constriction.

In another set of experiments, brain removal down to the pons, leaving the pituitary intact, was carried out (1), in addition to adrenal vein cannulation and inferior vena caval cuffing. Twenty-four hours following these three procedures, a protocol similar to that in dogs without central nervous lesions was carried out. In addition, the dogs with brain removal were given an intravenous dose of 20 units of corticotropin approximately six hours following the caval constriction experiment.

RESULTS

Results obtained following caval constriction in normal dogs with adrenal vein cannulas and vena caval cuffs are given in Table 1. The mean corticosteroid output in the basal or "resting" state for these six control animals was 1.4 μg./min. with a standard error of the mean of 0.3. Following vena caval constriction, a markedly significant increase ($P<.001$) in corticosteroid output was observed in all animals and the mean value obtained was comparable to that in previous studies from this laboratory following nerve stimulation or exogenous ACTH.

All six animals of this group demonstrated 20 to 40 mm. of mercury depression in arterial blood pressure during caval constriction. Pressures before constriction ranged from 100 to 140 mm. of mercury. The arterial pressure depression occured within three minutes following partial caval constriction, was sustained throughout the 45-minute period of constriction,

TABLE 1. CONTROL STUDIES IN NORMAL DOGS WITH ADRENAL VEIN CANNULAS AND VENA CAVAL CUFFS

Pre- and post-constriction values represent average of 3 samples obtained in the immediate period before and following caval constriction.

Experiment No.	Adrenal venous 17-hydroxycorticosteroids (micrograms/minute)	
	Pre-constriction	Post-constriction
EA-13	2.1	14.6
EA-12	2.3	16.2
EA-10	0.7	19.2
EA- 7	1.4	13.4
EA-17	1.1	17.9
EA-19	0.7	18.4
Mean	1.4 ±0.3*	16.6 ±0.9*

* Standard error of the mean.

TABLE 2. STUDIES IN DOGS WITH BRAIN REMOVAL, ADRENAL CANNULAS AND VENA CAVA CUFFS

Pre- and post-constriction values represent average of 3 samples obtained in the immediate period before and following caval constriction. ACTH values represent average of 2 samples obtained 5 and 10 minutes following 25 units ACTH intravenously.

Experiment No.	Adrenal venous 17-hydroxycorticosteroids (micrograms/minute)		
	Pre-constriction	Post-constriction	Post-ACTH
EA-16	9.8	7.6	13.8
EA-14	0.6	11.2	8.6
EA-11	7.0	12.0	17.5
EA- 9	6.3	10.3	9.5
EA- 6	4.7	18.5	15.4
Mean	5.7 ± 1.5*	11.9 ± 1.8*	13.0 ± 1.7*

* Standard error of the mean.

and pressures had returned to normal when post-constriction samples were obtained. Adrenal venous blood flows were unchanged in three animals and slightly increased in three others in samples obtained after release of caval constriction and return of venous pressure to normal. Venous pressures were increased 10 cm. of water from control levels of 2–6 cm. of water. Rectal temperatures remained constant throughout these experiments.

Early in the course of these experiments it was found that the minor surgical trauma of femoral arterial cannulation resulted in a significant increase in adrenal corticosteroid output in most animals. Adrenal venous outputs remained elevated for periods varying from 15 minutes to 2 hours. At least a 2-hour period must intervene between arterial cannulation and the control period because of temporary stimulation of pituitary adrenal activity, in order to ensure "basal" corticosteroid outputs.

Results following partial caval constriction in dogs with brain removal are given in Table 2. It can be seen that a significant elevation in the "resting"corticosteroid output is observed in these animals as compared to the normal ($P<.05, >.02$). In addition, the animals taken as a group revealed a significant increase following caval constriction ($P<.05, >.02$). There was no further increased output of corticosteroids following exogenous ACTH administration ($P<0.9$). The statistics would show a higher degree of significance if it were not for dog EA-14 which demonstrated a low "resting" level of corticosteroid output. Dog EA-14 is the only animal out of 25 brain removal experiments in which a low "resting" output of corticosteroids has been observed. A significant response following caval constriction was observed in this animal, as in 3 of the 4 other animals with brain removal.

Dog EA-16, which did not show an increase of corticosteroids following caval constriction, had a very high "resting" level prior to constriction. Adrenal venous blood flows remained either constant or became slightly elevated following caval constriction in these animals, as in the normals. Blood pressure before constriction ranged from 100 to 140 and blood pressure depressions of 20 to 60 mm. of mercury were observed following caval

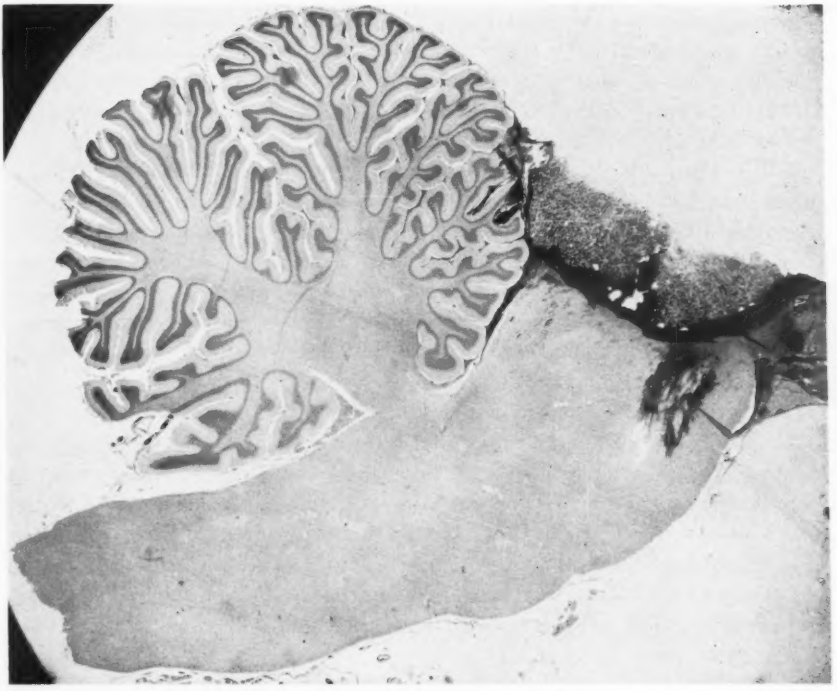

FIG. 1. Photomicrograph of sagittal section near mid-line of dog EA-14. (H&E. 13.5×) Note complete absence of all brain tissue above pons. The pituitary was intact in this animal, with a small sub-sellar remnant of hypothalamus present.

constriction. In all animals, the return of arterial blood pressure to normal was achieved within 10 minutes following release of caval constriction.

Study of serial sagittal sections of the brain stem and pituitaries of dogs with brain removal revealed intact pituitaries in all instances. No attempt was made in these experiments to remove the sub-sellar remnant of hypothalamus. Examination of the brain stem revealed removal of the brain down to the level of the pons, with no persistent mesencephalon (Fig. 1).

DISCUSSION

The findings of this study demonstrate clearly that partial thoracic inferior vena caval constriction resulting in an elevation of peripheral venous pressure of 10 cm. of water represents an effective adrenal cortical stimulus. Davis and co-workers (4, 5) have applied the technique of vena caval constriction in the study of aldosterone secretion, and have not demonstrated increases in corticosterone secretion following caval constriction. Although these findings appear to be at variance with those of the present report, there are several possible explanations for the discrepancy. Firstly, adrenal

vein cannulas were performed on the day of caval constriction in the aldosterone experiments. Results from our laboratory and those of others have demonstrated that a period of at least several hours is necessary for the elevated adrenal venous corticosteroid output to fall back to low levels following surgical trauma of this magnitude. As has been pointed out in the results of the present study, such a simple procedure as arterial cannulation often results in a significantly elevated corticosteroid output. Accordingly, it is most likely that the aldosterone experiments were performed in dogs already secreting maximal quantities of corticosterone and hydrocortisone. There is no assurance in such experiments in "acute" animals that increased outputs of endogenous ACTH in response to a given procedure will be reflected in increased corticosteroid output by an already maximally stimulated adrenal cortex.

It has previously been shown that the ratio of hydrocortisone to corticosterone in dog adrenal venous blood ranges from 3/1 to 6/1 (6, 7) and the average "basal" corticosterone outputs in the studies of Davis were approximately 3 μg./min. (Hydrocortisone was not measured). A hydrocortisone secretion of 9–18 μg./min. is within the range of maximal stimulus from operative trauma or exogenous ACTH in our laboratories. The results of the experiments described in the present paper make it clear that partial caval construction with peripheral venous pressure elevation represents a strong stimulus to ACTH secretion.

Dogs with brain removal and isolated pituitaries respond to caval constriction with an increased adrenal venous corticosteroid output. These dogs with brain removal are similar to a series of animals previously reported (1) with respect to the elevated basal corticosteroid outputs. Although small sub-sellar hypothalamic remnants were present in all specimens of the present studies, it has previously been shown that removal of this hypothalamic tissue does not prevent the high "resting" levels or response to nerve stimulation. A complete discussion concerning the possible mechanisms of pituitary ACTH secretion in dogs with isolated pituitaries has been given elsewhere (1), and the present study does not provide further evidence with which to elaborate on this discussion. It establishes, however, that higher central nervous system areas are not essential for the pituitary-adrenal activation which occurs following caval constriction. Inasmuch as the pituitary is relatively devoid of nerve supply, it is most likely that a neurohumor is produced in the region of the hind brain (HBF) which passes through the systemic blood stream to cause pituitary ACTH release. It is felt that high "resting" corticosteroid outputs in these animals occur because of removal of an inhibitory area to the hind brain, and that further increases of corticosteroid output occur following nerve stimulation or caval constriction based on increased output of HBF.

REFERENCES

1. Egdahl, R. H.: *Endocrinology* **66:** 200. 1960.
2. Hume, D. M. and D. H. Nelson: *Surg. Forum* **6:** 568. 1955.
3. Nelson, D. H. and L. T. Samuels: *J. Clin. Endocrinol. and Metab.* **12:** 519. 1952.
4. Davis, J. O., M. M. Pechet, W. C. Ball, Jr. and M. J. Goodkind: *J. Clin. Invest.* **36:** 689. 1957.
5. Yankopoulos, N. A., J. O. Davis, B. Kliman and R. E. Peterson: *J. Clin. Invest.* **38:** 1278. 1959.
6. Bush, I. E.: *Endocrinology* **7:** 210. 1954.
7. Farrell, G. L. and B. Lamus: *Proc. Soc. Exper. Biol. and Med.* **84:** 89. 1953.

ENZYMIC CORRELATES OF PHYSIOLOGICAL REGRESSION OF FOLLICLES AND CORPORA LUTEA IN OVARIES OF NORMAL RATS[1,2]

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ABSTRACT

Histochemical changes in the activity of two hydrolytic enzymes (acid phosphatase and aminopeptidase) considered to be characteristic of lysosomes were studied in ovarian structures. Ovaries from juvenile, adult cyclic, pregnant, lactating, and post-lactating rats were examined.

Healthy follicles exhibited almost no detectable enzymic activity. Whenever follicles appeared atretic, marked activity had developed in the follicular epithelium. Comparison of these preparations with sections prepared by routine staining methods suggested that a rise in enzymic activity may be indicative of incipient atresia. Similarly, an increased enzymic activity developed in lutein cells prior to the next ovulation, before shrinkage of the corpora lutea was detectable.

The results tend to support the hypothesis that these methods permit recognition of early destructive changes in follicles and corpora lutea, which in both cases may be hormonally determined. Contrariwise, no rise in activity occurred in oocytes of atretic follicles, even when these were undergoing lysis.

ATRESIA of follicles and involution of corpora lutea occur normally in cyclic ovarian activity. Both follicles and corpora are transiently-developed (2), and their regressions are examples of physiological autolysis. Biochemically, autolysis may be defined as the hydrolysis of endogenous cellular substrates, including proteins, polysaccharides, lipides, and organic esters (3). Such digestive activity may bring about the disappearance of parts of cells or of whole cells. In the last few years, it has been proposed that "lysosomes" may play a significant role in intracellular digestion, including autolysis (4–6).

Lysosomes are a recently-described class of cytoplasmic particles with which a number of hydrolytic enzymes are associated (3, 4). Morphologi-

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[2] A preliminary report was made at the Eleventh Annual Meeting of the Histochemical Society (1)

cally, they are described as being electron-dense particles with an average diameter of 0.3 to 0.5 μ (7). They possess a single membrane (6, 7). Biochemically, the membrane is semipermeable and effectively separates the enzymic complement within from the rest of the cellular constituents. These enzymes are easily-soluble hydrolases, having in common acid pH optima. In addition to acid phosphatase and cathepsins (intracellular proteases and peptidases) (3–6), a number of other hydrolases have been found associated with the lysosome fraction of liver homogenates (3, 4, 8–11).

Intracellular digestion has long been recognized to occur after such physiological activities as phagocytosis and pinocytosis. The association of lysosomes or lysosome-like particles with these processes has already been suggested or demonstrated (3, 5, 6, 12, 13). Digestive processes also occur during the regression and necrobiosis of cells (5, 6). The lysosomes, by virtue of their hydrolytic enzymes, would appear well-equipped to carry out these functions.

It has been proposed that the healthy cell may be protected from the destructive action of these hydrolases by the impermeability of the lysosomal membrane (4). The enzymes would thus be unable to act while this barrier is intact, because substrates are prevented from entering the lysosomes or the contained enzymes from leaving. However, if the membrane is altered in some way, it may become permeable to substrates, or, in reverse, to the contained enzymes.

The question then arises, what alters the lysosomal membrane to permit activity of the contained enzymes when autolytic processes begin? To date there have been few studies bearing on this question. De Duve and coworkers (4) have shown that these hydrolytic enzymes escape from the particles after the oxygen tension of an organ has been decreased. Moreover, Wolff (14) has studied the emergence of digestive activity in the Müllerian duct of developing male chick embryos. Proteolytic enzymes became active when endogenous or exogenous androgens were at a high level; this condition favors regression of the duct. Brachet *et al.* (15) have shown that when regression is occurring, the lysosomal enzymes studied are more soluble than earlier, suggesting that the membrane has become permeable.

Histochemically, some form of fixation plus other manipulations (e.g., freezing and thawing) of tissue appear to alter the properties of even intact lysosomes sufficiently so that the membrane becomes permeable to the substrates (16). It is important to emphasize that the histochemical method is appropriate only when the enzyme is somehow retained in the section. A truly soluble enzyme leaves the section and causes hydrolysis in the incubating medium; this product is precipitated diffusely rather than at the original sites of activity. A discussion of this question of desmo (bound) versus lyo (soluble) enzymes is to be found in Hannibal and Nachlas (17).

The presence of lysosomes (as indicated by histochemical reactivity) has

previously been described by Novikoff (5, 6) in many different physiological and pathological situations. Included was the observation that activity is high in demonstrably atretic follicles and in some of the corpora lutea of rat ovaries.

The study of incipient follicular atresia by morphological criteria has not proved satisfactory (18, 19). There is agreement in the literature that it is not possible to recognize early atresia by the use of routine histological methods. An earlier study indicated that lipide droplets appear in the granulosa when a follicle becomes atretic (20). We undertook the present study to determine whether the histochemical demonstration of hydrolytic enzymes could be used for earlier recognition of follicular atresia. It also seemed of interest to study just when enzyme activity develops in corpora lutea. These ends could be accomplished by removing ovaries at known periods of the estrous cycle and during pregnancy. The two enzymes here used as representative of lysosomes are acid phosphatase and aminopeptidase (5, 6). In addition to studies of follicles and corpora lutea, observations on oocytes are included.

MATERIAL AND METHODS

To provide a wide spectrum of conditions, rat ovaries were selected as follows. The normal cyclic animals were 24 young adult rats of the Sprague-Dawley strain, with an average weight of 180 gm. Vaginal smears were taken daily to establish the length and regularity of the estrous cycles, and only rats having regular cycles were used.

Rats were selected at the following stages. (a) *Metestrus* (11 rats), since at this stage there would be newly-formed corpora lutea, as well as corpora from the previous cycles; also any follicles that had failed to ovulate would be undergoing degeneration at this time. (b) *Diestrus* (9 rats), when medium-sized follicles, both healthy and atretic, are present, and the corpora of the most recent ovulation are fully developed. (c) *Proestrus* (4 rats), when there are a great number of large follicles about to ovulate, and the corpora of the current cycle have passed their maximum development.

Animals were also selected during early, mid, and late pregnancy (6 rats), during lactation (2 rats), and shortly thereafter (1 rat). The ovaries of juvenile rats (5 animals, 20 to 45 days of age) were likewise examined.

The rats were killed by decapitation. The ovaries were immediately placed into prechilled 10% formalin neutralized with an excess of sodium acetate, and then fixed overnight at 4° C. Frozen sections of the ovaries were cut at 10 μ.

For histological study, some sections were stained (a) with hematoxylin and eosin; (b) overnight with 10^{-3} M methylene blue, pH 4.5; and (c) with Oil red O (21). The reasons for our choice of these methods will be explained under Results.

To demonstrate enzymatic activity, other sections were incubated (a) by the Gomori acid phosphatase procedure (21), at pH 5.0, for 20 and 40 minutes; (b) after acetone extraction, by the aminopeptidase method of Burstone and Folk (22), using L-leucyl-2-naphthylamide HCl and Garnet GBC at pH 7.1, for 1 and 2 hours; and (c) by the Gomori alkaline phosphatase method (21), pH 9.4, 3 minutes. Control sections were incubated without substrates.

In addition to frozen sections from formalin-fixed organs, as described above, tissues prepared in other ways were tested. In all cases, sections were stained for acid phosphatase activity and adjacent sections were prepared by other methods necessary for

the identification of structures. (a) Sections of frozen, unfixed rat ovaries were cut in a cryostat at −20° C, mounted on coverslips and dried. These were used either unfixed or after fixation in 10% neutralized formalin or in calcium-formalin (5, 6) for 30 minutes, 4 hours, and 24 hours, at 4° C. (b) Sections of unfixed ovaries were cut in the cryostat, mounted and dried. They were then frozen and thawed 5 times (5 minutes at −20°, 5 minutes at room temperature, each) before incubation. (c) Ovaries fixed overnight in calcium-formalin at 4° were then transferred, according to Holt's procedure (16), to 0.88 M sucrose in 1% gum acacia and left for one week at 4° before use. Other ovaries were left in neutralized formalin for a week in the refrigerator. (d) Frozen sections from neutral formalin-fixed blocks were treated with the detergent Triton X-100 (16) for one-half hour, both at room temperature and at 4°. (e) Some sections cut from blocks fixed overnight as usual were mounted and dried on slides and left in the refrigerator overnight. Others of the same series were left in distilled water in the refrigerator overnight, then mounted on slides the next day. The two sets of sections were incubated together.

RESULTS

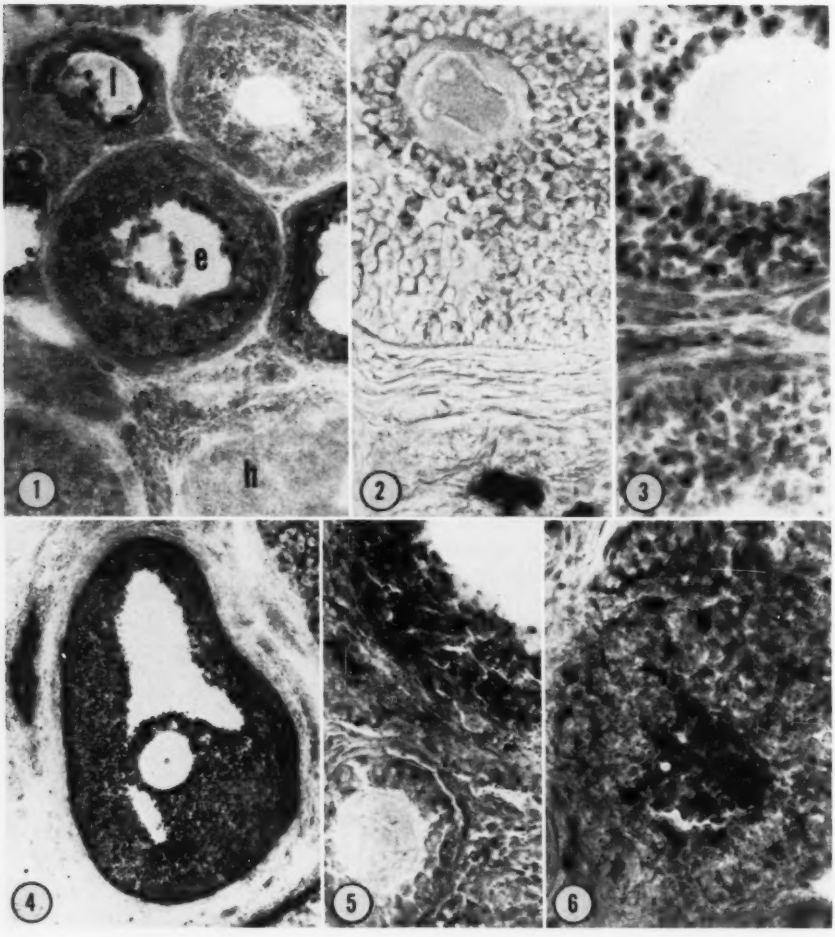
Follicles. For the initial identification of atretic follicles, we used the presence of lipide droplets in the granulosa cells (20) and various morphological features, such as distortion of the shape of the follicle (Figs. 4, 7, 8) and a ragged appearance of the follicular epithelium (Figs. 7, 8, 16, 19). A round, symmetrical follicle with regularly-arranged follicular epithelium and no detectable lipide was regarded as healthy. The subsequent study showed that many follicles not appearing abnormal by these criteria displayed enzymic properties identifying them as atretic.

Healthy follicles exhibited no detectable acid phosphatase or aminopeptidase activity in the granulosa cells (Figs. 1, 2, 5, 8, 16, 19). In any one section, some follicles had no reactivity in the granulosa, while others showed varying degrees of enzymic activity. Follicles recognizable morphologically as atretic by the criteria enumerated above always had a high degree of activity in this layer (Figs. 1–6, 10, 16, 17, 19). Some normal-appearing follicles also possessed activity (Figs. 3, 8, 9). From comparing histological preparations, especially those stained with Oil red O, with adjacent enzymic preparations, it was our impression that some follicles showing no detectable free lipide already showed an increase in enzymic activity.

In acid phosphatase preparations, the reaction appeared in granules (Figs. 3, 5, 10). As emphasized by Novikoff (5), when overall activity appeared weak, the individual granules were small (Fig. 2), whereas when reactivity became more intense, the granules appeared larger (Figs. 3, 5, 10). In aminopeptidase preparations, possibly because of the diffusibility of azo-dye products, the intracellular localization was not clear, but the reaction product was certainly in the cytoplasm.

An increase of activity of these enzymes was independent of the size of the follicle; follicles in all stages of growth possessed reactivity when undergoing atresia (cf. Figs. 3, 5, 8).

As atresia progressed there was an apparent dissolution of the cells in the



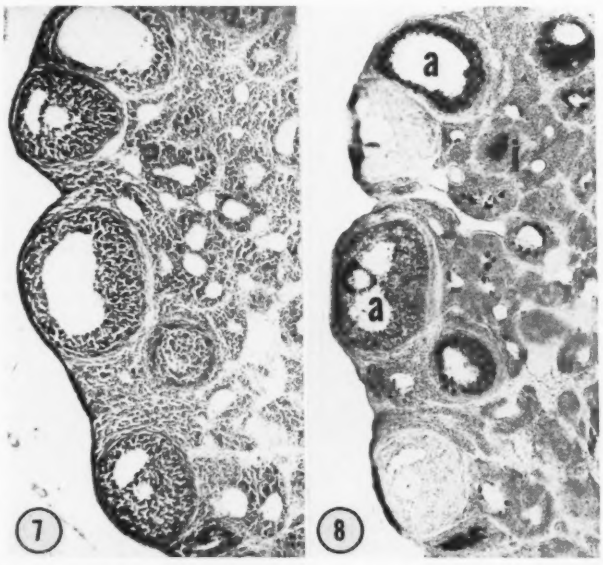
FIGS. 1–6. Examples of ovarian follicles in different physiological states, all incubated to demonstrate acid phosphatase activity. Figs. 1–4, 20-minute incubations; Figs. 5 and 6, 40-minute incubations.

FIG. 1. Juvenile rat (30 days old); ×ca. 110. Healthy follicle (h) and follicles in early (e) and late (l) stages of atresia. Reactive granules are absent from the healthy follicle and increasingly abundant as atresia progresses.

FIG. 2. Pregnant rat; ×ca. 320. The upper portion of the figure illustrates the edge of an apparently healthy follicle; only occasional small granules are detectable in the granulosa; none appears in the oocyte. The lower edge of the figure contains interstitial tissue with marked activity in the central detritus.

FIG. 3. Juvenile ovary; ×ca. 320. Segments of two follicles in early stages of atresia. There are more granules in the granulosa cells than in Fig. 2; again the oocyte shows no activity.

FIG. 4. Pregnant rat; ×ca. 80. A slightly distorted but otherwise normal-appearing



FIGS. 7 and 8. Nearly adjacent frozen sections of an ovary of a juvenile rat (30 days old); ×ca. 50. Fig. 7 stained with dilute methylene blue; Fig. 8 stained for acid phosphatase activity, 20-minute incubation. The 4 large follicles on the surface all appear essentially normal morphologically, but two of them have developed conspicuous acid phosphatase activity in the granulosa, and are deemed atretic (a). Many smaller follicles, some of which are detectably atretic, also show marked activity. Some lobules of interstitial tissue (i) retain central masses of degenerate granulosal cells (see Figs. 1, 2, 6, 12).

granulosa (Figs. 1, 6, 8, 11, 12). The amount of reactive material increased, and in the late stages of atresia there seemed to be a confluence of the granules (Figs. 2, 6, 12, 19). As known from earlier studies (20, 23), when the granulosa fragments and disappears, the follicle as a whole shrinks, but the cells of the theca interna persist and possibly proliferate. The center of the follicle, previously occupied by granulosa and fluid, is obliterated, and the atretic follicle is thus converted into a lobule of interstitial tissue. Some such lobules may still contain an oocyte (Figs. 11, 12) (20).

Even when entirely surrounded by degenerating cells, oocytes were generally devoid of any sign of hydrolytic enzymic activity (Figs. 1, 3, 4, 9, 10,

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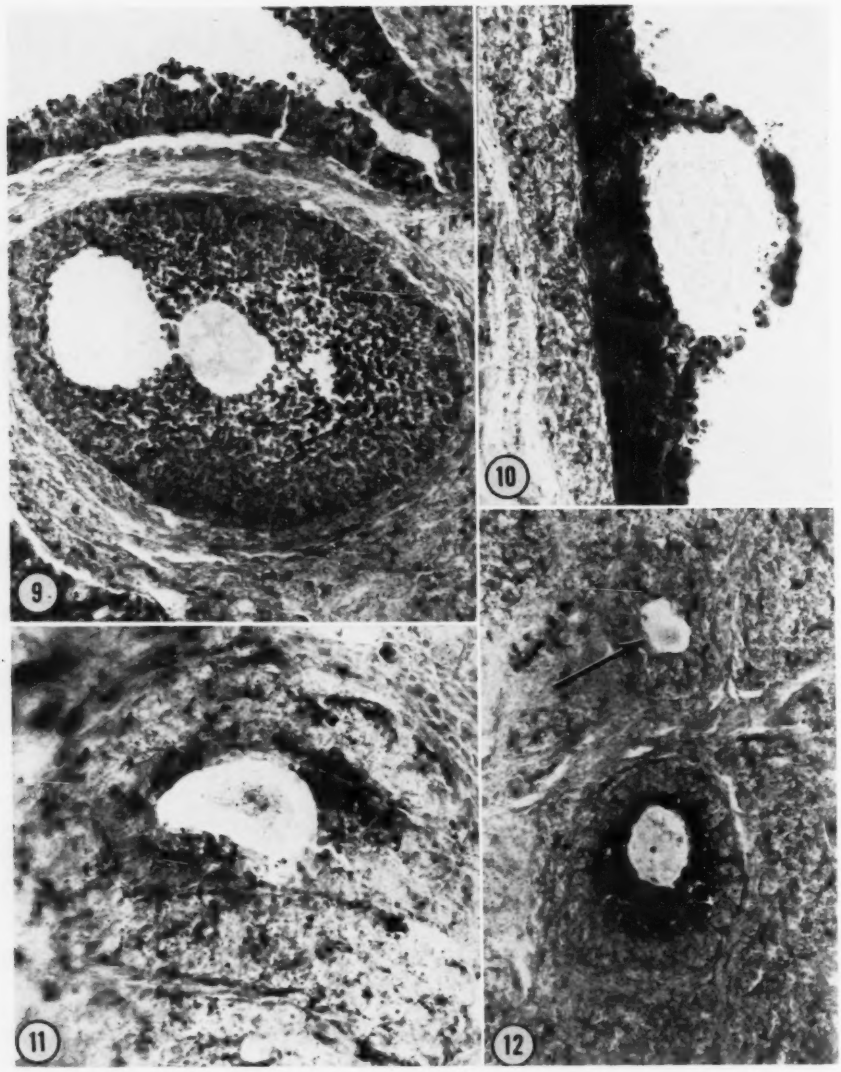
follicle, with very marked activity in the granulosa. The apparent activity in the nucleolus in the oocyte is a false-positive reaction (24).

FIG. 5. Diestrous rat; ×ca. 320. Above, the edge of a large follicle undergoing atresia; there is a conspicuous accumulation of reactive granules. There is virtually no granular activity in the wall of a very small follicle in the lower left.

FIG. 6. Diestrous rat; ×ca. 320. A section through a new lobule of interstitial tissue, with a central mass of degenerate granulosa cells exhibiting marked activity.



FIGS. 9–12. Oocytes within atretic follicles. All sections incubated to demonstrate acid phosphatase activity, 40 minutes.

FIG. 9. Diestrous rat; ×ca. 125. Small vesicular follicle. The oocyte, although vacuolated in appearance (lipide ?), shows no acid phosphatase activity.

FIG. 10. Juvenile rat (26 days); ×ca. 360. Cumulus oophorus of a large vesicular follicle shows marked activity in the granulosa.

FIG. 11. Metestrous rat; ×ca. 360. Follicle almost completely converted to interstitial tissue; all granulosa cells have disappeared, the oocyte exhibits very wea

12). In only one or two instances did the egg cell itself exhibit a fine granular precipitate, and then only when the follicle had been almost completely converted to a lobule of interstitial tissue (Fig. 11).

Corpora lutea during the estrous cycle. The corpora lutea were divided into the following classes: (a) those of the first generation (post-ovulatory); (b) corpora of the second generation (i.e., of the previous cycle); (c) still older corpora. The ages of these corpora were judged primarily by the degree of basophilia toward methylene blue and the size of intracellular lipide droplets (20). Corpora lutea of the first generation stained intensely with methylene blue and showed very fine and evenly-distributed lipide droplets (Figs. 13, 14). In metestrus, these corpora were not yet well-vascularized, as shown by the reaction for alkaline phosphatase (Fig. 15), which in the ovary is prominent in capillaries and larger vessels.

First generation corpora exhibited relatively little acid phosphatase and aminopeptidase activity in comparison with older corpora (Figs. 16, 17). Structurally, new corpora showed regular cords of small lutein cells (Fig. 20). As these corpora grew during diestrus, activity made its appearance but was limited almost completely to perivascular macrophages. Only occasional, small granules possessing acid phosphatase activity occurred in the lutein cells at this stage. The number of granules then seemed to increase during proestrus.

Corpora of the second generation (formed 4–8 days previously) were identifiable by a much weaker staining with methylene blue, and by the accumulation of large intracellular lipide droplets (Figs. 13, 14). In the sections incubated for acid phosphatase and aminopeptidase, such corpora exhibited very high reactivity (Figs. 16, 17). The enzymic reaction product occurred not only in pericapillary macrophages but also abundantly within lutein cells (Fig. 21). During their second generation, the lutein cells became increasingly necrotic in appearance.

Corpora of still earlier cycles had less enzymic activity. Most of the parenchymatous cells had disappeared by this time, but there was still a strong reaction in the connective-tissue phagocytes (Fig. 18).

Corpora lutea of pregnancy and lactation. During pregnancy the lutein cells became much larger than those in cyclic corpora (Fig. 22). By 10 days these corpora were no longer basophilic, but they possessed only small lipide droplets, like first generation corpora. During mid and late gestation, changes in the size and distribution of these droplets occurred; they en-

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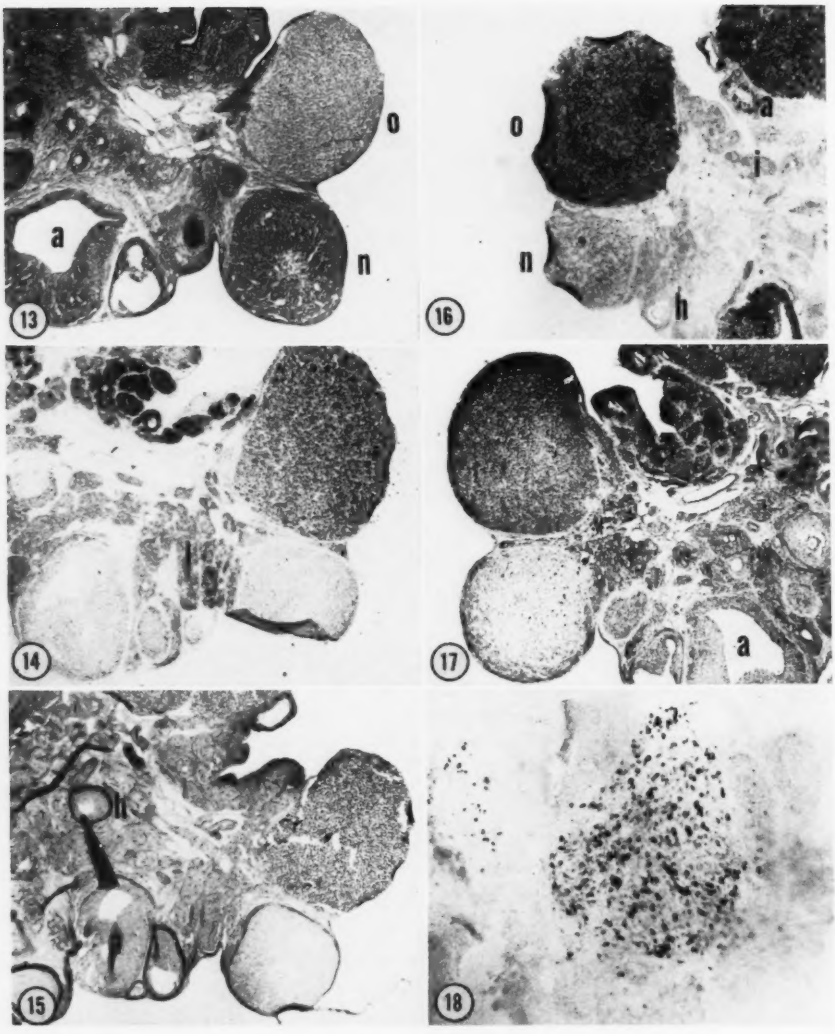
enzymatic activity.

FIG. 12. Diestrous rat; ×ca. 125. Two new lobules of interstitial tissue are present. The upper one (arrow) resembles that in Fig. 11, although the central residue (oocyte ?) exhibits no activity. The lower lobule still contains highly reactive granulosal debris; the oocyte possesses no activity (cf. Figs. 4, 6).



FIGS. 13–17. Adjacent sections of a rat ovary (metestrus), all ×ca. 20; (n) new corpus, (o) corpus of the preceding cycle, (a) luteinizing atretic follicle, (h) small healthy follicle, (i) lobule of interstitial tissue. Fig. 13. Stained with dilute methylene blue. The new corpus luteum is much more basophilic than the old one. The granulosa of the atretic follicle is also basophilic, as is this layer in young healthy follicles. Fig. 14. Stained with Oil red O. The new corpus and the atretic follicle contain little free lipide the old corpus exhibits large droplets. Lipides also occur in the interstitial tissue and the thecas of follicles. Fig. 15. Stained for alkaline phosphatase activity. The walls o blood vessels within the old corpus, in the theca surrounding the new corpus luteum, a well as in those of follicles, and in the interstitial tissue all react positively. In froze sections, unlike paraffin sections (20), there is little difference in reactivity of atretic an

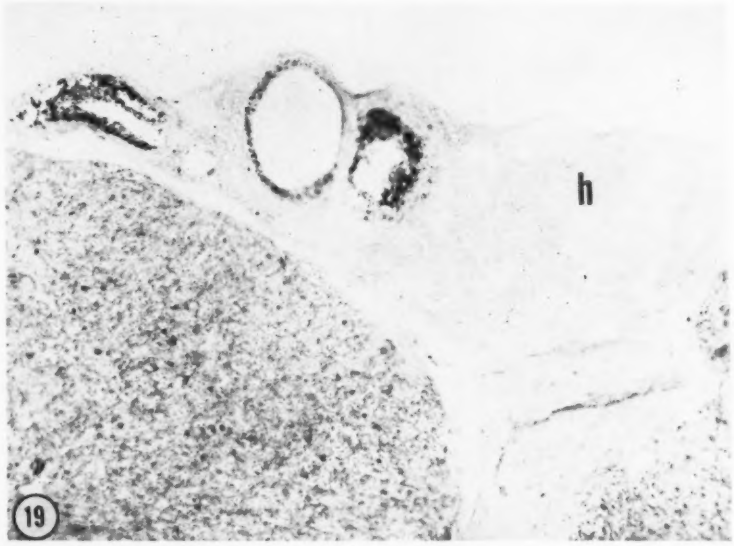


FIG. 19. Ovary of a pregnant rat (day 15), incubated for acid phosphatase activity, 20 minutes, ×80. Portions of 2 large corpora show only weak activity. Three severely atretic follicles at the top of the section exhibit marked activity in the granulosal layer, while a healthy small follicle (to the right of h) shows none.

larged in some lutein cells but not in others. The corpus as a whole remained about the same size, however (e.g., Fig. 19), and no shrinkage was evident until after parturition.

The corpora of pregnancy were well vascularized. They exhibited only slight aminopeptidase or acid phosphatase activity (Fig. 19). Some intracellular granules of enzymic reaction product were present in the lutein cells (Fig. 22); more were present in the pericapillary macrophages (not evident in this figure). Corpora lutea of early, mid and late pregnancy were not readily distinguishable from each other on the basis of hydrolytic enzymic activity as demonstrated by the methods used.

The new corpora formed after parturition became large, like those of

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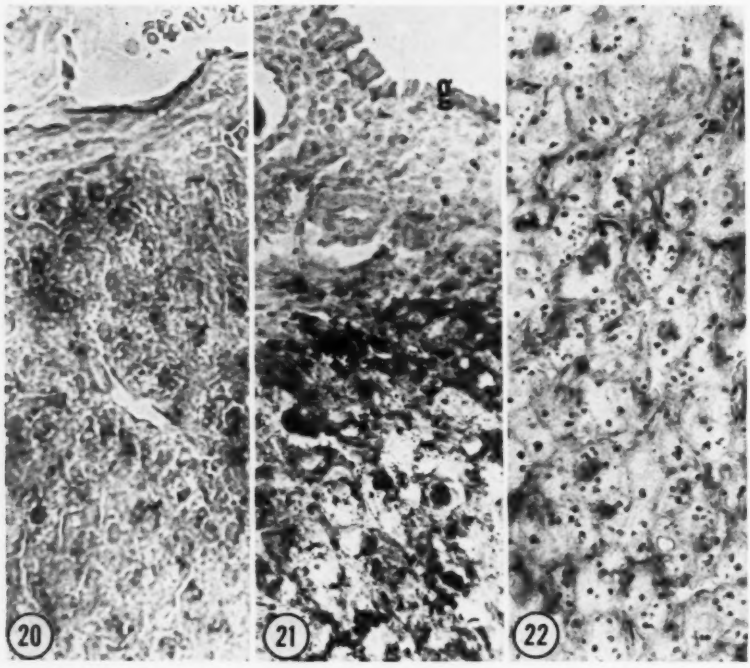
healthy follicles. Fig. 16. Stained for acid phosphatase activity, 20-minute incubation. The two old corpora and the atretic follicles display marked activity; the new corpus has little, and a small healthy follicle has none. Interstitial tissue is only slightly active. Fig. 17. Stained for aminopeptidase activity, 1-hour incubation. The reactivity is essentially parallel to that for acid phosphatase, although the luteinizing atretic follicle is relatively lighter.

FIG. 18. Section of the same ovary, stained for acid phosphatase activity, ×ca. 65. A severely regressed corpus luteum (? 3rd generation) shows marked activity only in the residual macrophages.



FIGS. 20–22. Portions of corpora lutea stained for acid phosphatase activity, ×400. Figs. 20 and 21, rats in metestrus; Fig. 22, pregnant rat (same as Fig. 19). In the new corpus luteum (Fig. 20) activity is almost nil. In the regressing corpus luteum (Fig. 21) marked activity occurs in phagocytes and also in lutein cells. Note that no activity occurs in the germinal epithelium (g). In the corpus luteum of pregnancy, one or two foci of activity occur per lutein cell.

pregnancy, and remained equally deficient in enzymic activity as long as lactation continued. However, in the ovary of the animal killed 4 days after lactation had terminated, the receding, large corpora had marked activity.

Other preparative methods. The experiments described to compare our standard procedure with other preparative methods yielded the following results.

(a) Sections that had been cut in the cryostat before fixation and then incubated for acid phosphatase activity, either unfixed or after fixation, were quite unsatisfactory. Totally-unfixed sections showed very uneven results, with some areas appearing unreactive, while some old corpora gave intense but diffuse reactions. If the sections had been fixed before incubation, the reaction for acid phosphatase was again variable and, where it occurred, generally diffuse. However, in some structures granules such as those described above were detectable.

(b) When unfixed cryostat sections were repeatedly frozen and thawed before incubation, no staining of cytoplasmic granules occurred. Where staining was present, there was a diffuse background wash plus some precipitation of the reaction product on nuclei (24).

(c) When fixed blocks were kept for a week in cold gum-sucrose (16), sections incubated for acid phosphatase showed quite poor localization of final reaction product. The granules were ill-defined, the surrounding cytoplasm showed more intense staining, and nuclear staining was also the general rule. Blocks kept in our usual fixative for a week had lost reactivity.

(d) Sections treated with the detergent Triton X-100, either at room temperature or in the refrigerator, exhibited marked loss of overall activity, and where some reaction product occurred it was diffusely distributed.

(e) When fixed sections that had been left in ice water overnight were incubated, little enzymic activity persisted, and the product was located on nuclei rather than in cytoplasmic granules (24). Sections that had been dried and kept in the refrigerator overnight exhibited staining limited almost entirely to cytoplasmic particles; no nuclear staining was evident.

DISCUSSION

In this report we have interpreted the enzymic activity observable within the granulosal cells of atretic follicles and in regressing lutein cells as demonstrating lysosomes, as suggested by Novikoff (5, 6). This interpretation is based in part upon the localization of the reaction in cytoplasmic granules, especially in the case of acid phosphatase. Further support for this concept was given by our studies dealing with pretreatment of tissue other than fixation with cold neutralized formalin and sectioning on a CO_2-chilled microtome. The experiments using unfixed cryostat sections suggested that freezing tissue before fixation damages the lysosomal membrane, so that much enzyme leaches out and is not available to be preserved by subsequent fixation. Freezing and thawing the sections before fixation permit even greater loss, as would be expected from the observations of de Duve (3, 4). Similarly, treatment of sections with a detergent, Triton X-100, allowed leaching out of enzyme, as has been demonstrated by Holt (16). Soaking fixed sections in water apparently had the same effect. We could not duplicate the beneficial effects on preservation of activity reported by Holt (16) of keeping fixed tissue blocks in an isotonic gum-sucrose mixture. Apparently acid phosphatase was partially solubilized under these conditions.

Follicular atresia. Some of the morphological changes that have been widely accepted as evidence of atresia are pyknosis of the nuclei of the granulosal cells and fragmentation of the cells bordering on the antrum, when the follicle is large, or around the ovum, when the follicle has not yet become vesicular. Also described have been shrinkage and distortion of the entire follicle and nuclear changes in the oocyte. Some workers have de-

scribed abnormal mitotic figures in the granulosa as indicative of the earliest sign of atresia (18). Generally these studies have been limited to clearly atretic follicles, and usually only to vesicular follicles (20, 25–29). No agreement exists as to which of the above changes is primary and all workers have stressed that atresia may have begun before such morphological changes were evident.

An earlier study on rat ovaries by one of us (20) suggested that histochemical changes may precede visible morphological alterations. Atretic follicles differed from normal ones in that some or all of the granulosa cells contained glycogen, lipide droplets and ascorbic acid, and exhibited alkaline phosphatase and nonspecific esterase activity. A subsequent study by Moss *et al.* (30) on cow ovaries confirmed some of these observations. The enzyme reactions were performed on paraffin sections, a method no longer deemed satisfactory because localization is unreliable (24). In fact, with frozen sections we have not been able to confirm the observation that alkaline phosphatase activity rises in atretic follicles.

The present results indicate that activity of the lysosomal enzymes acid phosphatase and aminopeptidase is detectable in the granulosal cells very early in the atretic process. By comparing sections stained for enzyme activity with adjacent one stained for lipides, it appeared that such activity rises before lipide droplets are demonstrable. This observation suggests that one of the earliest alterations in a follicle undergoing atresia may be an increase in the concentration of such hydrolytic enzymes. The activity of these enzymes may be responsible for the subsequent morphological changes characteristic of atresia.

We have no observations that would help explain why certain follicles develop high levels of activity of these enzymes and become atretic. The causal mechanism of atresia has not been determined. Sturgis (31), among others, has emphasized the possibility that atresia may be a result of circulatory embarrassment. In this respect one is reminded of de Duve's observations on the deleterious effects of low oxygen levels on the lysosomal membrane (4). Why circulatory failure occurs in some follicles, however, and not in others is still open to debate. Both mechanical (31) and hormonal factors (32, 33) have been invoked.

Two earlier reports become especially interesting in view of these observations that the granulosa cells of atretic follicles develop high titers of digestive enzymes. One is the demonstration by Evans (34) that these cells take up certain vital dyes when follicles become atretic. This suggests that one function of the granulosa cells in atretic follicles may be the removal of both follicular fluid and remnants of the oocyte. More recently, Hisaw and Hisaw (35) have reported that, in elasmobranchs, the follicular epithelium consumes the yolk when oocytes die. Phylogenetically, therefore, phagocytosis may have been a significant function of these cells. The

same enzymes used for digestion of phagocytized material would also be capable of digesting the cell's own protoplasm.

Our results conform with those of Sturgis (31), in that atresia was evident in young follicles without antra as well as in larger follicles. Furthermore, as emphasized earlier by Arai (36) and Slater and Dornfeld (37), a large number of follicles become atretic prior to the first ovulation in juvenile rats.

Destruction of oocytes. It is of particular interest that no hydrolytic enzymic activity demonstrable by our methods could be found within oocytes in even markedly atretic follicles. Others have reported (20, 38) that ova may appear normal despite advanced atretic changes in the granulosa. It is possible that oocytes, at least in the mammals studied, may not possess an autolytic mechanism such as that described here for granulosa cells. Indeed, Brachet *et al.* (15) have reported that lysosomes, according to the biochemical criteria established by de Duve, do not exist in the *amphibian* egg.

We have no direct explanation as to the mechanism bringing about dissolution of the oocyte so characteristic of advanced follicular atresia. Several possibilities may be suggested. Soluble hydrolases derived from the granulosa cells may reach the oocyte and subsequently cause its breakdown, or enzymes other than those we have studied may be responsible for its destruction. Evans (34) suggested that macrophages may be responsible for removal of the detritus seen within the cavity of the atretic follicle. It would be impossible to determine whether all of the reactive material in our preparations is within granulosa cells (Figs. 6 and 12); some reactive cells may indeed be macrophages.

Luteolysis. Earlier workers have described an accumulation of lipide followed by shrinkage of the cells as corpora lutea regress (20, 39–46). Ultimately there is nuclear pyknosis and fragmentation of the cells, central necrosis, and replacement of parenchyma by connective tissue. However, as with the follicle undergoing atresia, it has not been possible to pin-point the time at which regression begins in the corpus. Histochemically, aside from the accumulation of large lipide droplets containing demonstrable amounts of cholesterol (20, 44, 47), no marked differences distinguish the corpus beginning to shrink from the full-size one.

The present study suggests that a rise in acid phosphatase activity occurs in lutein cells when they begin to involute and that this activity remains high until all recognizable parenchyma has disappeared. For the rat, the development of activity in proestrus coincides with the time of accumulation of cholesterol-rich lipide droplets (20, 47). In corpora of pregnancy and lactation, there was no rise in enzymic activity with time. An increase appeared only after parturition in corpora of pregnancy, and after suckling had terminated in corpora of lactation.

For rodents, current evidence suggests that corpora lutea begin to involute when the titer of circulating luteotrophic hormone (LTH) falls in relation to the titer of luteinizing hormone (LH, ICSH) (47, 48). In the normal cycle, this shift coincides with the rising production of estrogen by the new crop of follicles, and Klein (49) has suggested that a high titer of estrogen, rather than the decline in LTH, may be responsible for the involution. Whichever hormonal shift is causative, it appears that luteolysis, apparently initiated by the rising amount of hydrolytic enzymes, is governed by hormones. More certainly than is the case for follicular atresia, luteolysis thus resembles the involution of the Müllerian duct in chick embryos (14).

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REFERENCES

1. Rosenbaum, R. M., B. L. Lobel and H. W. Deane: *J. Histochem. & Cytochem.* **8:** 321. 1960. (Abstract)
2. Zuckerman, S.: *Ciba Foundation Colloquia on Ageing* **2:** 31. 1955.
3. de Duve, C.: *Exp. Cell Res.* **Suppl. 7:** 169. 1959.
4. de Duve, C.: In "Subcellular Particles," Ed. T. Hayashi, Ronald Press, New York, p. 128, 1959.
5. Novikoff, A. B.: In "Developing Cell Systems and Their Control," Ed. D. Rudnick, Ronald Press, New York, p. 167, 1960.
6. Novikoff, A. B.: *Biol. Bull.* **117:** 385. 1959.
7. Novikoff, A. B., H. Beaufay and C. de Duve: *J. Biophys. & Biochem. Cytol.* 2, **Suppl.:** 179. 1956.
8. Paigen, K. and S. Griffiths: *J. Biol. Chem.* **234:** 299. 1959.
9. Sellinger, O., H. Beaufay, P. Jacques, A. Doyen and C. de Duve: *Biochem. J.* **74:** 450. 1960.
10. Conchie, J., J. Findlay and G. A. Levvy: *Biochem. J.* **71:** 318. 1959.
11. Viala, R. and R. Gianetto: *Canad. J. Biochem. & Physiol.* **33:** 839. 1955.
12. Bennett, H. S.: *J. Biophys. & Biochem. Cytol.* **2, Suppl.:** 185. 1956.
13. Straus, W.: *J. Biophys. & Biochem. Cytol.* **5:** 193. 1959.
14. Wolff, E.: *Experientia* **9:** 121. 1953.
15. Brachet, J., M. Decroly-Briers and J. Hoyez: *Bull. Soc. Chim. Biol.* **40:** 2039. 1958.
16. Holt, S. J.: *Exp. Cell Res.* **Suppl. 7:** 1. 1959.
17. Hannibal, M. J. and M. M. Nachlas: *J. Biophys. & Biochem. Cytol.* **5:** 279. 1959.
18. Vincent, W. S. and E. J. Dornfeld: *Am. J. Anat.* **83:** 437. 1948.
19. Mandl, A. M. and S. Zuckerman: *J. Endocr.* **6:** 426. 1950.
20. Deane, H. W.: *Am. J. Anat.* **91:** 363. 1952.
21. Gomori, G.: "Microscopic Histochemistry," Univ. of Chicago Press, 1952.
22. Burstone, M. S. and J. E. Folk: *J. Histochem. & Cytochem.* **4:** 217. 1956.
23. Aron, M. and C. Aron: *Arch. Anat. (Strasb.)* **36:** 69. 1953.
24. Deane, H. W., R. J. Barrnett and A. M. Seligman: *Handbuch der Histochemie* **VII/1.** 1960.
25. Allen, E., J. P. Pratt, Q. U. Newell and L. J. Bland: *Am. J. Anat.* **46:** 1. 1930.
26. Kingsbury, B. F.: *Am. J. Anat.* **65:** 309. 1939.

27. Williams, P. C.: *Ciba Foundation Colloquia on Ageing* **2:** 59. 1955.
28. Mandl, A. M. and S. Zuckerman: *J. Endocr.* **13:** 262. 1956.
29. Block, E.: *Acta Endocr.* **8:** 33. 1951.
30. Moss, S., T. R. Wren and J. F. Sykes: *Anat. Rec.* **120:** 409. 1954.
31. Sturgis, S. H.: *Carneg. Inst. Contrib. Embryol.* **33:** 67. 1949.
32. Paesi, F. J. A.: *Acta Endocr.* **11:** 251. 1952.
33. Ingram, D. L., *J. Endocr.* **19:** 123. 1959.
34. Evans, H. M.: *Anat. Rec.* **10:** 264. 1916. (Abstract)
35. Hisaw, F. L., Jr., and F. L. Hisaw: *Anat. Rec.* **135:** 269. 1959.
36. Arai, H.: *Am. J. Anat.* **27:** 405. 1920.
37. Slater, D. W. and E. J. Dornfeld: *Am. J. Anat.* **76:** 253. 1945.
38. Nelson, W. W. and R. R. Greene: *Am. J. Obst. & Gynec.* **76:** 66. 1958.
39. Corner, G. W.: *Carneg. Inst. Contrib. Embryol.* **31:** 117. 1945.
40. Boling, J. L.: *Anat. Rec.* **82:** 131. 1942.
41. Brewer, J.: *Am. J. Obst. & Gynec.* **44:** 1084. 1942.
42. Baillif, R. N.: *Am. J. Anat.* **83:** 109. 1948.
43. Harrison, R. J.: *Biol. Rev.* **23:** 269. 1948.
44. White, R. F., A. T. Hertig, J. Rock and E. Adams: *Carneg. Inst. Contrib. Embryol.* **34:** 55. 1951.
45. Barker, W.: *Endocrinology* **46:** 772. 1951.
46. Corner, G. W., Jr.: *Am. J. Anat.* **98:** 377. 1956.
47. Everett, J. W.: *Endocrinology* **41:** 364. 1947.
48. Greep, R. O.: *Endocrinology* **23:** 154. 1938.
49. Klein, M.: *Schweiz. Med. Woch.* **79:** 503. 1949.

EFFECTS OF METHYL GROUP SUBSTITUTION FOR IODINE ON METABOLIC ACTIVITY OF THYROXINE, TRIIODOTHYRONINE AND TETRAIODOTHYROPROPIONIC ACID[1]

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ABSTRACT

Compared to 3′,5′,3,5-tetraiodothyropropionic acid, 3′,5′-dimethyl-3,5-diiodothyropropionic acid was 15 to 36% more active as measured by tissue metabolism or basal metabolic rate, respectively, of injected thyroidectomized rats. Similarly, 3′,5′-dimethyl-3,5-diiodo-DL-thyronine approached 44% of the activity of L-thyroxine and 3′-methyl-3,5-diiodo-L-thyronine was 44% as active as 3′,3,5-L-triiodothyronine on an equimolar basis. In contrast 3′,5′,3,5-tetramethyl-DL-thyronine displayed no detectable metabolic activity.

AMONG the many alterations in the thyroxine molecule carried out in recent years, substitution of other halogens for the iodines on the diphenyl ether nucleus has been most common. In general, bromine and chlorine replacement of iodine has lowered physiological activity in that order (1–3). Tetranitrothyronine showed very slight thyroxine-like stimulation of metabolism, although it resulted in considerable growth in young thyroidectomized animals (4). Four methyl-containing thyroxine-like materials: 3′,5′-dimethyl-3,5-diiodo-DL-thyronine (Me_2T_2), 3′,5′,3,5-tetramethyl-DL-thyronine (Me_4T), 3′-methyl-3,5-diiodo-L-thyronine (MeT_2), and 3′,5′-dimethyl-3,5-diiodothyropropionic acid (Me_2Pr_2)[2] have been evaluated, since, from some physico-organic aspects, methyl groups might be expected to out-perform iodines (5). Only very small amounts of the first two compounds named above were available.

METHODS

Sprague-Dawley albino rats, at least 6 weeks post-thyroidectomy, were used exclusively. Metabolic responses were evaluated both on whole animals, by measuring the

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[2] We are indebted to Dr. Jan Wolff, National Institute of Health, Bethesda, Md., for 3′,5′-dimethyl-3,5-diiodothyronine (synthesized by Glaxo) and tetramethylthyronine (synthesized by Bielig and Lützel, 19). Dr. N. Kharasch kindly provided tetraiodothyropropionic acid, 3′,5′-dimethyl-3,5-diiodothyropropionic acid and 3′-methyl-3,5-diiodothyronine. L-Thyroxine was furnished by Dr. H. A. Fevold, Travenol division of Baxter Laboratories. The 3,5-L-diiodothyronine was a commercial sample.

post-absorptive metabolic rate, and on a variety of tissues, with oxygen consumption measurements in the Warburg apparatus. For the first, a modified Watts and Gourley procedure (6) was used for Basal Metabolic Rate (BMR) determinations before and during injection of the compounds being tested, in some instances a commercially available servo-activated apparatus.[3] The animals were placed on a special feeding regimen (7) to insure a post-absorptive condition for determination of the BMR. After the animals had been trained to the apparatus, at least three series of activity-free BMR's were obtained. L-Thyroxine (T_4), 3′,3,5-L-triiodothyronine (T_3) or the desired analogue was then injected subcutaneously once per day for each of 4 days. BMR's were measured after 2, 3 and 4 days of injecting and the highest two averaged; a plateau was nearly always reached after 3 and 4 days.

For injections, T_4, T_3 or the desired analogue was dissolved in the proper amount of 0.1N NaOH to make 0.02N final NaOH concentration when diluted with 0.9% NaCl to make the appropriate dosage. Each compound was injected subcutaneously once per day, dosages being shown as micromoles/kg./day.

For tissue metabolism, control and analogue-injected animals were anesthetized with ether after 4 days' injections, exsanguinated and the desired tissues placed in chilled Krebs' Ringer solution. Oxygen consumption runs were made in Krebs' Ringer-phosphate-glucose at 37° (8, page 149) using "intact-cell" preparations. Slices of liver, kidney cortex, salivary gland, pancreas and cardiac muscle (left ventricle) were cut using the Deutsch technique (8, page 136), with Schick blades. The entire diaphragm was removed, the central tendon carefully cut out and quarter-diaphragms prepared by cutting parallel to the visible muscle fibers. Bundles of skeletal muscle fibers were teased by blunt dissection from the long parallel-fibered anterior neck muscles using the technique of Richardson, Shorr and Loebel (9).

RESULTS

In contrast to the results on tadpole metamorphosis (10), 3′,5′,3,5-tetraiodothyropropionic acid (Tetraprop) is not a metabolically potent compound in warmblooded animals. As measured by whole animal BMR, Tetra-prop is only 5% as active as L-thyroxine (T_4) and 14% by summated oxygen consumption of several different organ tissues (11, 12). Our results in Table 1 show that 3′,5′-dimethyl-3,5-diiodothyropropionic acid (Me_2Pr_2) was slightly more active than its tetraiodinated analogue, Tetraprop. Replacement of iodines by methyl groups at the 3′,5′-positions increased Tetraprop activity by 36.2% in the BMR experiments and 15.4% in the tissue runs. This is not the prominent difference obtained with amphibia (10, 13).

The di- and tetra-methylated thyronines could be tried in one or two animals only, and the data on whole animal BMR and tissue metabolism were obtained from the same animals (Table 2). 3,5-Diiodo-L-thyronine was only partially active even at high dosage, in both BMR and tissue experiments. With addition of methyl groups at 3′,5′-positions to produce 3′,5′-dimethyl-3,5-diiodo-DL-thyronine (Me_2T_2), the compound became distinctly activated although there was considerable disparity between the effects on BMR and tissue metabolism, 17% and 71% respectively. The striated muscle, as here represented by diaphragm, showed a much lower

[3] Obtainable from Custom Engineering and Construction Co., St. Louis, Mo.

TABLE 1. EFFECTS OF 3′,5′,3,5-TETRAIODOTHYROPROPIONIC ACID (TETRAPROP) AND 3′,5′-DIMETHYL-3,5-DIIODOTHYROPROPIONIC ACID (Me_2Pr_2) ON BMR AND TISSUE METABOLISM OF THYROIDECTOMIZED RATS

	% change from controls	
	Tetraprop 7.74[1]	Me_2Pr_2 7.74[1]
BMR (4 rats/group)	+22.1 100%	+30.1 136.2%
Tissue metabolism (6 rats/group)		
Diaphragm	+23.3	+25.6
Liver	+31.3	+39.8
Kidney	+17.8	+31.4
Salivary Gland	+29.4	+34.8
Pancreas	+39.1	+26.1
Heart	+38.6	+47.8
Skeletal Muscle	+15.9	+20.0
Average	+27.9 100%	+32.2 115.4%

[1] Dosage as micromoles/kg./day injected subcutaneously.

metabolic response than the cardiac muscle to most of the thyroxine-like compounds. In actual percentage of body weight, the total bulk of striated muscle is about 50% and that of heart only 0.4% (14). Therefore, if corrected for actual weight in the total animal, the summated tissue response to Me_2T_2 was probably closer to 45% than 71% as active as L-thyroxine. Since the DL-form of Me_2T is being compared to L-T_4, the above activities might become twice as high. For the present, we prefer to use the lower values.

3′,5′,3,5-Tetramethyl-DL-thyronine was unexpectedly found to possess very little thyroxine-like activity. As shown in Table 2, it was first tried unsuccessfully at 1.29 μM./kg. Even at a dosage of 7.74 μM./kg., BMR

TABLE 2. COMPARISON OF METABOLIC EFFECTS OF L-THYROXINE (T_4), 3,5-DIIODO-L-THYRONINE (3,5-T_2), 3′,5′-DIMETHYL-3,5-DIIODO-DL-THYRONINE (Me_2T_2) AND 3′,5′,3,5-TETRAMETHYL-DL-THYRONINE (Me_4T)

Compound	T_4		3,5-T_2	Me_2T_2	Me_4T	
Dose[1]	0.32	1.29	25.8	2.58	1.29	7.74
No. of animals	2	6	2	1	4	1
BMR						
% change	+14.3		+ 3.0	+19.9	+ 4.9	+10.3
% T_4 effect	100		0[2]	17.3	0[2]	0[2]
Tissue metabolism, % change from control						
diaphragm		+19.8	+19.4	+15.0	−14.0	+10.0
liver		+36.7	+14.6	+59.8	+ 3.3	+ 4.9
kidney		+35.9	+20.6	+36.5	+ 1.7	− 4.0
saliv. gl.		+32.9	+25.4	+32.1	—	− 6.5
heart		+38.6	+19.8	+87.9	− 9.9	+11.6
Average		+32.8	+20.0	+46.3	− 4.7	+ 3.2
% T_4 effect		100	3.0	70.6	0[2]	0[2]

[1] All doses expressed as micromoles compound injected subcutaneously/kg./day.
[2] Any metabolic change of 10% or less is arbitrarily considered zero.

TABLE 3. COMPARISON OF METABOLIC EFFECTS OF L-THYROXINE (T_4), 3′,3,5-L-TRIIODOTHYRONINE (T_3), AND 3′-METHYL-3,5-DIIODO-L-THYRONINE (MeT_2) IN THYROIDECTOMIZED RATS

Reference Compound:	T_4		T_3	
Dose[1]	0.65		0.13	
No. of animals	2		3	
BMR % change	+46.5		+49.5	
Dose of MeT_2[1]	0.13	0.26	0.13	0.26
No. of animals	6	6	6	6
BMR % change	+24.7	+37.0	+24.7	+37.0
% reference cpd	265.6	198.9	49.9	37.4

[1] All doses expressed as micromoles/kg./day injected subcutaneously.

and tissue oxygen consumption of the animals showed essentially no difference from control values.

From Table 3, it can be seen that 0.13 and 0.26 μM. doses of 3′-methyl-3,5-diiodo-L-thyronine (MeT_2) produced marked increases in BMR. Calculated out, MeT_2 was over twice as active as T_4. Comparison with T_3 is more valid, and on this basis the two doses of MeT_2 were 50 and 37%, respectively, as potent as T_3.

DISCUSSION

Studies of thyroxine analogues in recent years have done much to elucidate the relationship between molecular structure and biologic specificity (5, 15), yet no one hypothesis has stood all tests. In 1951 Lipmann and Dutoit postulated that thyroxine-like activity of the thyronine molecule was enhanced by substitution of an electron-withdrawing group such as nitro (16). Studies on halogen analogues of thyroxine already showed that this hypothesis was not correct (1–3), since iodine was the weakest electron withdrawer. Niemann (17) had earlier presented the hypothesis that thyroxine could function as an electron donor, going over to a quinonoid form. However, no oxidation-reduction systems have ever been found to incorporate thyroxine as carrier. Lissitzky (17) has shown convincingly that polyphenols and quinones can be produced from thyroxine, but these apparently are irreversible steps in oxidative degradation.

Table 4 summarizes our results with all thyroxine-like compounds reported here. It is apparent that iodines in positions 3 and 5 in combination with one 3′-methyl or two 3′,5′-methyls impart a considerable amount of

TABLE 4. SUMMARY OF METABOLIC ACTIVITIES OF FIVE METHYL-SUBSTITUTED THYROXINE-LIKE COMPOUNDS

Analogue	Reference	BMR[1]	Tissue[1]	Average
Me_2Pr_2	Pr_4	136	115	126
Me_2T_2	T_4	17	71	44
MeT_2	T_4	232	—	(232)
MeT_2	T_3	44	—	(44)
3,5-T_2	T_4	0	3	2
Me_4T	T_4	0	0	0

[1] Calculated as % of reference activity from results in Tables 1, 2 and 3.

metabolism-stimulating power to the thyronine molecule. As has been demonstrated many times before (15), the 3,5-iodines alone are inadequate. On the other hand, our experiment indicates that the iodine-replacing action of methyls at 3′ and 5′ does not extend to positions 3 and 5. These results agree well with those reported by Bruice *et al.* (5) for amphibia-tested compounds carrying alanine or amine side-chains.

These observations shed some light on the specific contributions of the "inner" benzene ring towards biological activity of thyroxine. Size alone of the 3,5-substituents cannot be limiting because the methyl group is nearly the same as the iodine. On the other hand, the iodo group is electron-attracting and exhibits a high degree of hydrogen-bonding, both characteristics in contrast to the methyl (5). The two iodines at 3,5, together with the ionizable side-chain, may be involved in some specific type of bonding with protein in the cell, a function which either one iodine alone or two methyls cannot perform. Since either iodine (electron-withdrawing, hydrogen-bonding) or methyl (electron-releasing, no hydrogen-bonding) substitution at 3′ or 3′ and 5′ can be effective, it is doubtful that electronic effects or hydrogen-bonding could be critical for the net contribution of the outer ring. These differences suggest that the function of substitutions on the two rings cannot be considered qualitatively similar.

REFERENCES

1. Leblond, C. P. and B. Grad: *J. Pharmacol. & Exp. Ther.* **94:** 125. 1948.
2. Lerman, J. and C. R. Harington: *J. Clin. Endocrinol.* **9:** 1099. 1949.
3. Mussett, M. V. and R. Pitt-Rivers: *Metabolism* **6:** 18. 1957.
4. Dutoit, C. H.: In "Phosphorus Metabolism," Vol. 2, ed. McElroy, W. D. and B. Glass, Johns Hopkins Press, Baltimore, Md.; 1952, p. 597.
5. Bruice, T. C., N. Kharasch and R. J. Winzler: *Arch. Biochem. & Biophys.* **62:** 305. 1956.
6. Watts, D. T. and D. R. H. Gourley: *Proc. Soc. Exper. Biol. & Med.* **84:** 585. 1953.
7. Barker, S. B.: *Proc. Soc. Exper. Biol. & Med.* **72:** 198. 1949.
8. Umbreit, W. W., R. H. Burris and J. F. Stauffer: *Manometric Techniques;* 3rd ed., Burgess Publishing Co., Minneapolis, Minn. (1957).
9. Richardson, H. B., E. Shorr and R. O. Loebel: *J. Biol. Chem.* **86:** 551 (1930).
10. Bruice, T. C., R. J. Winzler and N. Kharasch: *J. Biol. Chem.* **210:** 1. 1954.
11. Stasilli, N. R., R. L. Kroc and R. I. Meltzer: *Endocrinology* **64:** 62. 1959.
12. Hill, S. R., Jr., S. B. Barker, J. H. McNeil, J. O. Tingley and L. L. Hibbett: *J. Clin. Invest.* **39:** 523. 1960.
13. Roth, P. C. J.: *Ann. d'Endocrinol.* **19:** 1157. 1958.
14. Donaldson, H. H.: *The Rat;* 2nd ed., chapters 4, 7. Wistar Inst. of Anat. and Biol. Philadelphia, Pa., 1924.
15. Selenkow, H. A. and S. P. Asper, Jr.: *Physiological Reviews* **35:** 426, 1955.
16. Lipmann, F. and C. H. Dutoit: *Science* **113:** 474. 1951.
17. Niemann, C. and C. E. Redmann: *J. Am. Chem. Soc.* **63:** 1549. 1941.
18. Lissitzky, S. and S. Bouchilloux: *Bull. Soc. Chim. Biol.* **39:** 1215. 1957.
19. Bielig, H. S. and G. Lützel: *Ann. Chem.* **608:** 140. 1957.

THYROID FUNCTION IN THE RAT DURING PREGNANCY AND LACTATION[1]

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ABSTRACT

1) Pregnant rats were fed 0.15% propylthiouracil (PTU) from the 10th to the 19th day of pregnancy and 1, 2, 3, 4 or 5 μg. T_4 were injected daily during this time. The regression slopes of the thyroid weights were similar to those of the corresponding controls except that 2 μg. T_4 produced less decrease in thyroid size in pregnant than in non-pregnant rats. 2) Pregnant rats were fed a low-iodine diet from the 10th to the 19th day of pregnancy and doses of T_4 were administered during this interval as in 1). The regression slopes of the thyroidal I^{131} uptake of the pregnant and non-pregnant rats were identical but uptakes were consistently slightly lower at each point for the pregnant rats. 3) Following seven days of low-iodine diet, thyroidal I^{131} uptakes were determined at one to two day intervals from the 8th day of gestation to the 29th day of lactation. Uptakes were not statistically different from the controls until the 20th day of pregnancy when a marked drop of approximately 50% occurred which persisted throughout lactation. This decrease was correlated with a concomitant sudden rise in iodine uptake by the mammary glands. The high uptake by the mammary glands persisted throughout lactation and always exceeded that of the thyroid. Fetal thyroid uptake was negligible until the 20th day of pregnancy and then was only $\frac{1}{4}$ that of the mother. There was no consistent change in thyroid weight.

These results indicate that there is not a significantly increased demand for thyroid hormone during pregnancy in the rat. However, the marked decrease in radioiodine uptake by the thyroid during lactation seems related to the successful competition of the mammary glands for iodine during this time.

IT HAS long been held that goiter commonly appears in pregnant women (1, 2, 3), although some clinicians have noted that thyroid enlargement arises after delivery rather than during gestation (4). Although an elevation in plasma protein-bound iodine (PBI) (5, 6, 7) and in plasma thyroxine-binding capacity (8, 9) has been found in pregnancy and although there have been scattered reports of increased radioiodine uptake by the human thyroid during pregnancy (10, 11, 12, 13), comprehensive studies of thyroid function during gestation and lactation have not been made. It has been generally assumed that the increased thyroid size in pregnancy is similar to that seen during puberty, both being reflections of the increased

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need for thyroid hormone during these periods of accelerated physiologic activity. This hypothetical increased need for thyroid hormone has never been experimentally demonstrated however.

We considered it of interest to study this problem in some detail and therefore compared the following parameters of thyroid function in pregnant and nonpregnant rats: (1) the amount of thyroxine required to prevent goiter produced by chronic propylthiouracil (PTU) treatment; (2) the amount of thyroxine necessary to suppress thyroidal radioiodine uptake. In addition, I^{131} uptake and thyroid size were serially measured during pregnancy and lactation in a fairly large series of animals.

MATERIALS AND METHODS

Approximately 300 young, adult, pregnant Holtzman rats and their non-pregnant controls were utilized. Pregnancy was timed from detection of sperm on daily vaginal smear. To obtain an estimate of the amount of thyroxine required to prevent goitrogenesis, a portion of the animals was divided into five groups. These were fed 0.15% PTU from the 10th to the 19th day of pregnancy and 0, 2, 3, 4 or 5 μg. of thyroxine were injected daily during this period. On the 20th day of pregnancy, they were killed and their thyroid glands were dissected and weighed. This experiment was repeated with 0, 1, 2 and 3 μg. of thyroxine to extend the results of the first experiment.

To determine the amount of thyroxine required to depress radioiodine uptake, another batch of rats was divided into six groups and injected with graded doses (0, 1, 2, 3, 4 or 5 μg.) of thyroxine from the 10th through the 19th day of gestation. A low iodine diet was fed for seven days before radioiodine administration on the 20th day of gestation. Animals were killed four hours after the intraperitoneal injection of 0.1 μC. of I^{131}, and the thyroid glands dissected and weighed. Thyroidal radioactivity was determined with a well-type scintillation counter.

To detect any change in radioiodine uptake and thyroid size during pregnancy and lactation, four-hour I^{131} uptake and thyroid weight were measured at one to two day intervals from the 8th day of gestation to the 29th day of lactation. At the same time the radioiodine uptakes by the fetal or suckling thyroids and, in some instances, by the mammary glands were determined. The number and weight of the fetuses in each litter were determined. Litter size was reduced to six immediately after delivery. In some cases litter size was subsequently further reduced by death or infanticide. Suckling was continued until autopsy. I^{131} content of the mammary glands was determined by carefully dissecting all mammary tissue and counting it in a well-type scintillation counter. Again, all animals were fed a low iodine diet for seven days before radioiodine studies.

RESULTS

The amount of thyroxine required to prevent goiter produced by chronic PTU treatment

The regression slope of the thyroid weights of the pregnant rats was similar to that of the corresponding controls except that 2 μg. of thyroxine produced less decrease of thyroid size in the pregnant than in the non-pregnant rats (Fig. 1). Minimum thyroid weight was reached with 4 μg. of thyroxine daily with both the pregnant and non-pregnant groups. Since only two pregnant animals were available for the 2 μg. thyroxine group, the same

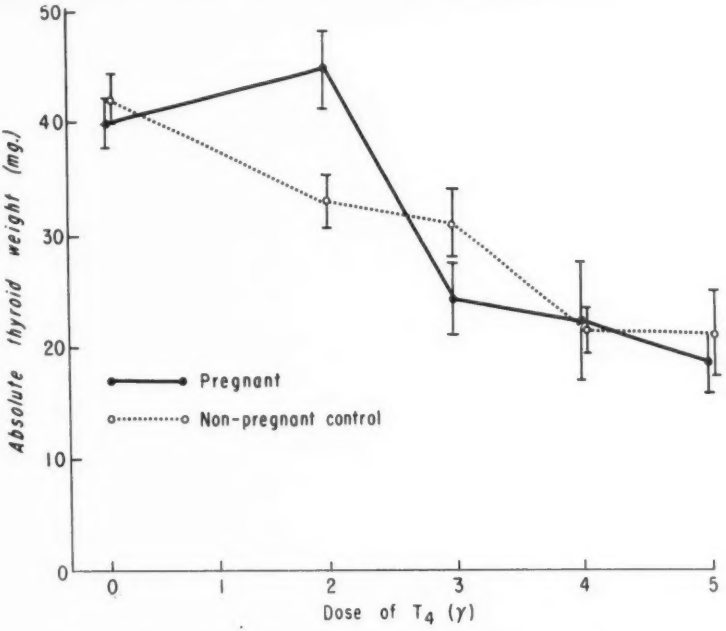

FIG. 1. Effect of thyroxine on thyroid weight in PTU-fed pregnant rats (First experiment). Animals fed 0.15% propylthiouracil for 10 days (pregnant group 10th through 19th day of gestation, autopsied on the 11th day. Each point and vertical line represents the mean and standard error of 6 to 10 animals, except that only 2 animals are included in the 2 μg. pregnant group. Indicated dose of thyroxine injected daily.

experiment was repeated with lower doses (0–3 μg.) of thyroxine. As can be seen in Figure 2, 2 μg thyroxine induced less regression of thyroid size in the pregnant group than was observed in the non-pregnant one.

The amount of thyroxine necessary to suppress thyroidal radioiodine uptake

The regression slopes of the radioiodine uptakes of the pregnant and non-pregnant rats treated with graded doses of thyroxine were identical, but the uptake values were consistently slightly lower at each point in the pregnant rats (Fig. 3).

Changes in I^{131} uptake and thyroid size during pregnancy and lactation

Uptakes were not statistically different from the controls (average 22% of dose) until the 20th day of pregnancy, when there was a sudden marked drop of approximately 50%. This decreased uptake persisted throughout lactation (Fig. 4). Fetal thyroidal uptake was negligible until the 20th day of pregnancy and then only reached 25% of that of the mother. Suckling uptakes were always very low. There was no consistent change in thyroid

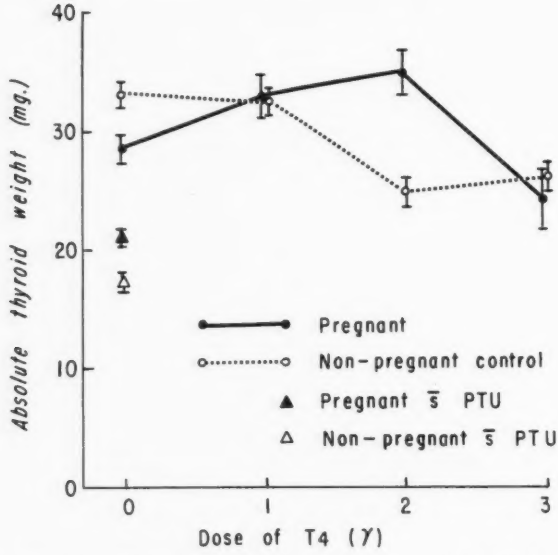

FIG. 2. Experiment as in Fig. 1. Each point represents 6 to 10 animals.

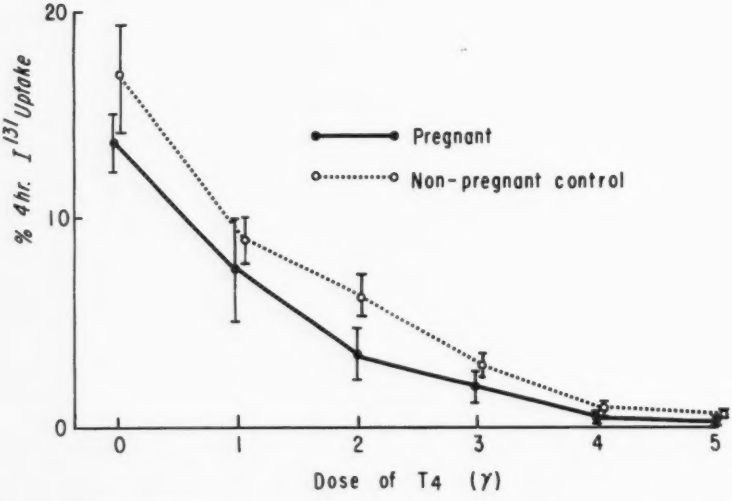

FIG. 3. Effect of thyroxine on thyroidal I^{131} uptake in the pregnant rat. Animals injected daily with indicated amount of thyroxine for 10 days (pregnant group 10th through 19th day of gestation) and autopsied on 11th day. Low-iodine diet fed for 7 days before autopsy. Each point represents at least 6 animals.

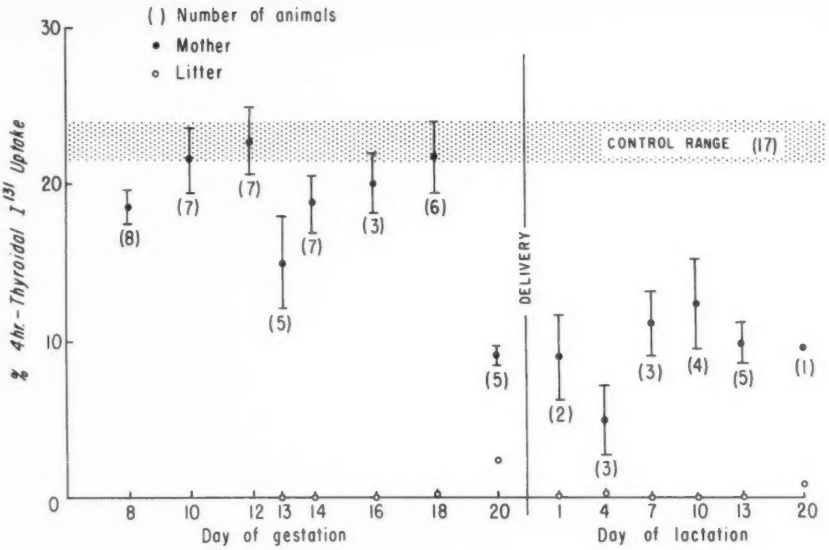

FIG. 4. Thyroidal I^{131} uptake during pregnancy and lactation. All animals fed low-iodine diet for 7 days before autopsy. Litter uptakes are the sum of all fetuses or sucklings.

weight throughout the experimental period (Fig. 5). No correlation was found between any of the parameters measured and the size or weight of the litter.

It is of considerable interest that the lactating mammary glands contained more radioiodine at 4 hours than did the thyroid gland (Table 1). In one rat (No. 92), who devoured her pups shortly after delivery, a 4-hour

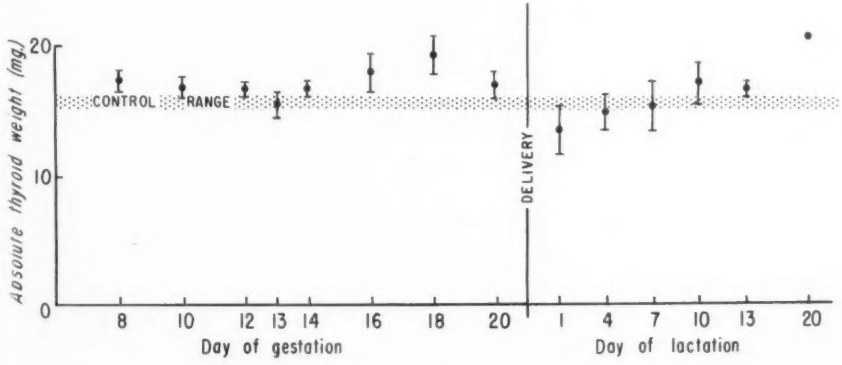

FIG. 5. Thyroid weight during pregnancy and lactation. Absolute thyroid weight is used to avoid errors in relative thyroid weight due to varying mass of fetuses.

TABLE 1. 4 HOUR RADIOIODINE UPTAKE BY THYROID AND MAMMARY GLANDS DURING LACTATION

Rat	Days postpartum	No. in litter	Percentage of administered dose		
			Maternal thyroid	Mammary glands	Litter thyroids
25	7	3	12.4	12.6	0.04
49	10	6	6.8	14.7	0.08
85	13	5	9.9	34.0	0.13
86	13	3	7.6	33.7	0.20
74	20	5	9.6	21.2	0.99
27	29	6	14.5	15.6	0.10
92	4*	0	26.1	0.92	—

* This rat devoured her offspring shortly after delivery.

radioiodine uptake on the 4th postpartum day was 26% in the thyroid but less than 1% in the mammary glands. Conversely, in one mother (No. 27) who was allowed to continue with her litter in the same cage eight days beyond the normal weaning period, thyroidal uptake was 14.5% while mammary gland uptake was 15.6%. This persistence of high radioiodine uptake by the mammary glands occurred in spite of the fact that the litter was eating solid food during much of this time, although nursing intermittently.

DISCUSSION

Exogenous thyroxine depresses thyroid activity chiefly by inhibiting the secretion of thyrotropin by the pituitary. It is generally accepted that the amount of thyroid hormone required to prevent thyroid enlargement produced by chronic antithyroid drug administration or to inhibit radioiodine uptake is proportional to metabolic demands for thyroid hormone by the animal. During periods of increased need, such as exposure to a cold environment (42), larger amounts are necessary to decrease thyrotropin secretion. It is assumed that the quantity of exogenous thyroxine required to prevent PTU-induced goitrogenesis or to depress radioiodine uptake to the hypophysectomy level is approximately equivalent to the endogenous secretion of thyroid hormone.

The present results indicate that there is no increased utilization of thyroid hormone during pregnancy in the rat since the amount of thyroxine required to prevent PTU-induced goiter or to depress radioiodine uptake was approximately equal in both pregnant and non-pregnant rats. Maternal radioiodine uptake also did not change during the period of gestation until the last day before parturition, while thyroid weight remained relatively constant throughout the experimental period.

Why 2 μg. of thyroxine produced less decrease of thyroid size in pregnant than non-pregnant PTU-treated rats is not clear at present. However, it seems unlikely that it was due to any physiologic alteration in the pregnant animals since the thyroid weight regression slopes were otherwise similar in

the pregnant and control groups. Although this difference is statistically significant ($P = 0.01$), it is likely that it is more accidental than real.

The fetal thyroid did not appear to accumulate appreciable amounts of I^{131} until just before delivery. This would coincide with the finding by Gorbman and Evans (14) that the fetal thyroid in the rat begins to accumulate iodine about the 19th day of intrauterine life.

The marked decrease in maternal thyroidal radioiodine uptake which began on the 20th day of pregnancy almost certainly was correlated with the concomitant sudden rise in iodine uptake by the mammary glands. This high uptake persisted throughout lactation and always exceeded that of the thyroid.

It has been observed previously that injected radioiodine is secreted into the milk in man (15, 16, 17) and in animals (18, 19, 20), and, in consequence, the I^{131} uptake by the thyroid gland is decreased (19). Brown-Grant studied this problem further in the rabbit and observed a rapid fall in I^{131} uptake after delivery and an accelerated thyroid secretion during the last week of gestation and during lactation (20). Our finding in the rat is therefore in good agreement with Brown-Grant's observation. As briefly mentioned by Potter *et al.* (21), it seems reasonable that with the sudden competition for iodine by the mammary glands, particularly if borderline iodine deficiency exists, increased thyroid activity will be required to preserve normal levels of thyroid hormone. This would thus explain why so frequently women notice thyroid enlargement following delivery and during the period of nursing rather than during pregnancy and why the apparent rate of thyroidal secretion increased during this period (20).

Some investigators have found that I^{131} uptake by the thyroid gland in man is higher throughout pregnancy (13) or in late pregnancy (11) and returns to normal after delivery (13). Pochin (10) studied the same problem and obtained similar but somewhat different results, indicating that the neck:thigh ratio is probably increased above normal during pregnancy, and decreased below normal soon after delivery. However, there was not a high degree of statistical difference ($P = 0.04$) in comparison of this ratio during pregnancy with that of normal controls, whereas the fall after delivery relative to the values during pregnancy was of considerably higher significance ($P = 0.002$). As to the fall in the thyroidal I^{131} uptake after delivery, neither of these investigators paid much attention to the role of the mammary gland in this stage. Extrapolating from the results of our study to another species, thyroidal I^{131} uptake might not actually increase during human pregnancy. It is possible that the relatively high thyroidal uptake observed during human pregnancy may have seemed absolutely high only when compared to the low uptake following delivery. The low thyroidal postpartum uptake may have been due to an increased accumulation of I^{131} by the mammary glands.

The physiologic significance of iodine concentration by the mammary

gland is not known. Some of the radioiodine accumulated by the mammary gland has been suggested (19) or proved to be protein-bound (22, 23). Various investigators have since found that the major components are iodide and monoiodotyrosine (21, 24) and a small amount of protein-bound I^{131} in the form of diiodotyrosine (21). In some instances, a thyroxine-like substance has been found (24), but it is not believed that thyroxine is formed in the mammary gland itself.

The iodine is secreted into the milk and thus made available to the suckling. One usually thinks of milk secretion as occurring at or shortly following parturition, but there is evidence of secretory activity during the second half of pregnancy (25, 26). For instance, Jeffers (27) found that the mammary alveoli of the rat suddenly became distended with secretion on the 20th day of pregnancy. This would correlate well with our finding that the thyroidal radioiodine uptake drops on the 20th day.

In the experiment in which graded doses of thyroxine were used to suppress radioiodine uptake, it was found that the thyroidal uptakes of the pregnant rats were slightly lower at each point than those of the non-pregnant animals. This might be explained by beginning iodide accumulation of the mammary glands in the pregnant animals, since these studies were completed on the 20th day of gestation.

It is well known there is a marked increase in estrogen and progesterone levels during pregnancy (28). In man, it has been shown that increased estrogen will cause a significant rise in the level of protein-bound iodine (29, 30) and it has been inferred that thyroid function is concomitantly increased. Some investigators have found in the rat that thyroidal uptake of I^{131} (31, 32) and the thyroid:serum radioiodine concentration ratio (33) are increased during estrus and that administration of estrogen (32, 34, 35) will increase thyroidal I^{131} uptake. However, estrogen administration in our laboratory (36) has not produced any consistent change in thyroidal radioiodine uptake. Our findings are thus similar to those of Paschkis *et al.* (37) and Dowling *et al.* (38). The rise in PBI during human pregnancy or during estrogen administration may be due solely to physico-chemical alteration of the thyroxine-binding capacity of serum protein (39, 40, 41).

Thus there does not seem to be a significantly increased demand for thyroid hormone during pregnancy in the rat. However, the marked decrease in radioiodine uptake by the thyroid during lactation seems related to the successful competition of the mammary glands for iodine during this time. Thus one might expect that thyroid enlargement in the mother noted by other observers during the period of nursing is related to relative iodine deficiency due to the increased loss of iodine from the body rather than to any specific need for increased thyroid hormone.

In these studies no thyroid enlargement was seen during lactation. It should be remembered, however, that all animals were on a low iodine diet

for seven days before autopsy and all thyroid weights were approximately 20–25% greater than those of rats on our standard laboratory diet.

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REFERENCES

1. Marine, D.: *Physiol. Rev.* **2:** 521. 1922.
2. Mussey, R. D.: *Am. J. Obst. & Gynec.* **36:** 529. 1938.
3. Freedberg, I. M., M. W. Hamolsky and A. S. Freedberg: *New England J. Med.* **256:** 505. 1957.
4. Marine, D.: *Glandular Physiology and Therapy*, ed. 3, Philadelphia, J. B. Lippincott, 1935, p. 345.
5. Heinemann, M., C. E. Johnson and E. B. Man: *J. Clin. Invest.* **27:** 91. 1948.
6. Danowski, T. S., R. C. Gow, F. M. Mateer, W. C. Everhart, S. Y. Johnston and J. H. Greenman: *Proc. Soc. Exper. Biol. & Med.* **74:** 323. 1950.
7. De Mowbray, R. R. and A. Tickner: *Lancet* **2:** 511. 1952.
8. Dowling, J. T., N. Freinkel and S. H. Ingbar: *J. Clin. Endocrinol.* **16:** 280. 1956.
9. Robbins, J. and J. H. Nelson: *J. Clin. Invest.* **37:** 153. 1958.
10. Pochin, E. E.: *Clin. Sc.* **11:** 441. 1952.
11. Noble, M. J. D. and S. Rowlands: *J. Obst. & Gynaec. Brit. Emp.* **60:** 892. 1953.
12. Ferraris, G. M. and A. Scorta: *Minerva ginec.* **7:** 308. 1955.
13. Halnan, K. E.: *Clin. Sc.* **17:** 281. 1958.
14. Gorbman, A. and H. M. Evans: *Endocrinology* **32:** 113. 1943.
15. Nurnberger, C. E. and A. Lipscomb: *J.A.M.A.* **150:** 1398. 1952.
16. Honour, A. J., N. B. Myant and E. N. Rowlands: *Clin. Sc.* **11:** 447. 1952.
17. Miller, H. and R. S. Weetch: *Lancet* **269:** 1013. 1955.
18. Courrier, R., J. Roche, G. H. Deltour, M. Marois, R. Michel and F. Morel: *C. R. Soc. Biol. (Par.)* **143:** 599. 1949.
19. Rugh, R.: *J. Morphol.* **89:** 323. 1951.
20. Brown-Grant, K.: *J. Physiol.* **131:** 70. 1956.
21. Potter, G. D., W. Tong and I. L. Chaikoff: *J. Biol. Chem.* **234:** 350. 1959.
22. VanMiddlesworth, L., A. H. Tuttle and A. Threlkeld: *Science* **118:** 749. 1953.
23. Brown-Grant, K.: *J. Physiol.* **135:** 644. 1957.
24. Brown-Grant, K. and V. A. Galton: *Biochim. et biophys. acta* **27:** 422. 1958.
25. Velardo, J. T.: *The Endocrinology of Reproduction*, New York, Oxford University Press, 1958, p. 222.
26. Linzell, J. L.: *Physiol. Rev.* **39:** 534. 1959.
27. Jeffers, K. R.: *Am. J. Anat.* **56:** 257. 1935.
28. Venning, E. H.: *Am. J. Med.* **19:** 721. 1955.
29. Engstrom, W. W., B. Markardt and A. Liebman: *Proc. Soc. Exper. Biol. & Med.* **81:** 582. 1952.
30. Engstrom, W. W. and B. Markardt: *J. Clin. Endocrinol.* **14:** 215. 1954.
31. Soliman, F. A. and E. P. Reineke: *Am. J. Physiol.* **178:** 89. 1956.
32. Feldman, J. D.: *Endocrinology* **58:** 327. 1956.
33. Boccabella, A. V. and R. G. Stuelke: *Endocrinology* **66:** 135. 1960.
34. Money, W. L., L. Kirschner, L. Kraintz, P. Merrill and R. W. Rawson: *J. Clin. Endocrinol.* **10:** 1282. 1950.
35. Florsheim, W. H.: *Am. J. Physiol.* **193:** 408. 1958.
36. Unpublished data.

37. Paschkis, K. E., A. Cantarow and W. C. Peacock: *Proc. Soc. Exper. Biol. & Med.* **68:** 485. 1948.
38. Dowling, J. T., S. H. Ingbar and N. Freinkel: *J. Clin. Endocrinol.* **19:** 1245. 1959.
39. Dowling, J. T., N. Freinkel and S. H. Ingbar: *J. Clin. Endocrinol.* **16:** 1491. 1956.
40. Robbins, J. and J. E. Rall: *Recent Progr. Hormone Res.* **13:** 161. 1957.
41. Engbring, N. H. and W. W. Engstrom: *J. Clin. Endocrinol.* **19:** 783. 1959.
42. Dempsey, E. W. and E. B. Astwood: *Endocrinology* **32:** 509. 1948.

EFFECTS OF HYPOTHALAMIC LESIONS ON THE SECRETION AND STORAGE OF HYPOPHYSIAL LUTEINIZING HORMONE

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ABSTRACT

Hypothalamic lesions in the median eminence of the tuber cinereum produced a syndrome of constant vaginal diestrus (CD), accompanied by atrophic ovaries and uteri and a significant decrease in weight of the pars distalis. The ovaries of these rats were characterized by failure of follicular development and the persistence of large corpora lutea. Lesions in the rostral hypothalamus, on the other hand, produced a syndrome of constant vaginal estrus (CE), accompanied by atrophic ovaries, and enlargement of uteri and pars distalis. The ovaries of these rats were filled with large follicles, and there was a scarcity of corpora lutea or their remnants.

Using the ovarian ascorbic acid depletion method, LH was measured in plasma and pars distalis before and after ovariectomy in normal rats and those with hypothalamic lesions. No detectable LH was found in plasma from normal rats, but detectable quantities of the hormone were found one to 16-weeks post-ovariectomy. Hypothalamic lesions producing CD or CE prevented this elevation of plasma LH following ovariectomy. Pituitary LH content was diminished to 33% of normal in rats with CE but was only 15% of normal in rats with CD. Ovariectomy produced a rise in pituitary LH content in normal rats and those with CE, whereas this response was blocked in rats with CD. It was concluded that the hypothalamus exerts a regulatory influence over synthesis and secretion of hypophysial LH.

THAT the hypothalamus exerts a regulatory influence over the secretion of gonadotrophins by the pituitary gland is supported by evidence obtained with various experimental approaches in a variety of species (1).

Two distinct syndromes related to gonadotrophin secretion can be produced in adult female rats by hypothalamic lesions. In one, the ovary is characterized by failure of follicular development and the persistence of functional corpora lutea, while the vaginal smear is continuously of the diestrous type. This syndrome is here referred to as Constant Diestrus (CD) and is produced by lesions destroying the median eminence (2). In the second syndrome, the ovaries contain large, often cystic follicles with little or no evidence of corpora lutea, indicating a failure of ovulation. The

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vaginal smear remains in the estrous phase for long intervals of time. This syndrome is designated Constant Estrus (CE) and is produced by hypothalamic lesions in a variety of loci, the most effective area lying in the suprachiasmatic region (3–7).

Little is known concerning the secretion rate of hypophysial gonadotrophins in these two syndromes. The CD animal secretes luteotrophin (LTH) as evidenced by positive deciduoma responses and lobuloalveolar development of the mammae (2). Secretion of follicle stimulating hormone (FSH) is thought to be low since follicular development is held in abeyance (2). Estrogen secretion appears to be deficient in these rats as well (8); however, virtually nothing can be inferred about the secretion rate of LH in CD rats since FSH must act on the ovary in order to obtain effects such as estrogen secretion and ovulation from luteinizing hormone (LH) administration (9).

The CE rat is obviously secreting some FSH as judged by the development of large follicles. The presence of a cornified vaginal smear is indicative of estrogen secretion which cannot occur in rats in the absence of LH secretion (9). Consequently, it is assumed by most workers that this type of animal secretes at least some LH but cannot release sufficient quantities to induce ovulation.

The ovarian ascorbic acid assay for LH developed by Parlow (10) provided us with a convenient and sensitive tool with which to study both secretion and hypophysial storage of LH in rats with hypothalamic lesions associated with either CE or CD. A preliminary report of this work has already appeared (11).

METHODS

Preparation of Assay Animals. Female rats, twenty-six days old, of the Sherman strain were pre-treated with gonadotrophins to produce heavily luteinized, enlarged ovaries. To accomplish this they were injected initially with 75 International Units (I.U.) of pregnant mare's serum gonadotrophin[3] subcutaneously (s.c.) and fifty-six hours later with 30 I.U. of human chorionic gonadotrophin s.c. Five to seven days thereafter the rats were used for assay of LH.

Technique of Assay of LH. The rats were anesthetized with ether and the left ovary was removed. Its ascorbic acid concentration represented the control, pre-assay value. The test solution to be assayed was then injected intravenously (I.V.) during a period of one minute, and one hour later, again under ether anesthesia, the second ovary was removed and anzlyzed for ascorbic acid concentration. Ascorbic acid was determined by a modification of the method of Mindlin and Bulter (12). The percentage depletion (decrease) in concentration of ascorbic acid in the second gland as compared to the first constituted the response.

Assays were performed on pars distalis of the hypophysis and on plasma. The pars distalis was collected, weighed on a torsion balance, and then ground with a stirring rod and a few grains of sand in 0.5 ml. of saline. Pools of 5–8 glands were diluted to

[3] The pregnant mare's serum gonadotrophin was a gift from Dr. J. B. Jewell of Ayerst Laboratories, 22 East 40th Street, New York 16, N. Y.

volume of 1 ml. per pituitary and centrifuged. Suitable dilutions of the supernatant extract were made with saline, so that 1 ml. of the diluted extract was injected into each assay rat. The pituitaries were assayed at two appropriate dose levels with a 5-fold concentration difference between doses. Two batches of pituitaries, one from ovariectomized and the other from non-ovariectomized donors, were assayed in a single lot of assay rats, and the results expressed in terms of the standard LH[4], which was run simultaneously at two dose levels. In each case five assay rats were run at each dose level.

For assay of plasma, donor rats were anesthetized with ether and 5–8 ml. of blood were withdrawn from the external jugular vein during 1–2 minutes time into heparinzed syringes. Following centrifugation, plasma from a pooled sample was assayed in recipients by the ovarian ascorbic acid depletion method. Two ml. of plasma were injected into each recipient rat. Since only a single dose of plasma was used, we have expressed these results in terms of ascorbic acid depletion rather than converting the figures into a value in terms of the LH standard.

Types of Donor Animals. All rats were adult, virgin females of the Sherman strain. Hypothalamic lesions were positioned either in the median eminence of the tuber cinereum to induce CD, or in the mid-line suprachiasmatic region to induce CE by methods previously described. Rats shown to be in either constant diestrus, or constant estrus by the vaginal smear record were chosen for experiments. The presence of an estrous smear at any time prior to use was sufficient cause for rejection of a CD rat, whereas CE rats were required to be continually in estrus for a period of at least two weeks prior to use. Ovariectomy was performed on a group of normals and on groups of rats with CD or CE as a results of hypothalamic lesions. Plasma from ovariectomized rats was assayed after the operation at various time intervals ranging from 1 week to 4 months. Pituitary LH was determined 2–3 months after placing the lesions, and one month after ovariectomy if this operation was performed.

Tissue Studies. Serial sections of the hypothalamus were made in all rats with lesions and every tenth section stained by the chrome alum hematoxylin-phloxin method (13). In a number of cases where the pituitaries were not used for assay of LH, they were serially sectioned and examined for infarcts. Ovaries and selected mammary glands were also sectioned and stained with hematoxylin and eosin.

RESULTS

Plasma LH. No detectable LH was found in plasma from normal female rats, in that there was no significant ovarian ascorbic acid depletion in the recipient animals (Fig. 1). Within a week following ovariectomy, plasma samples induced a significant depletion of ovarian ascorbic acid in the assay animals. This elevated concentration of LH persisted for at least four months post-ovariectomy without further significant increment.

In contrast to the results in rats without lesions, no detectable LH was found in the plasma of rats with hypothalamic lesions on assay one to five weeks post-ovariectomy (Fig. 1, 2). The results in the rats with CD were identical with those obtained in the rats with CE (Figs. 1, 2).

Pituitary LH Content. Analysis of pituitary LH content in pooled normal glands yielded reproducible values on four separate occasions (Table 1). One month following ovariectomy, pituitary LH was significantly elevated

[4] The LH standard was Luteinizing Hormone, Ovine (NIH-LH-S-1) and was a gift from the Endocrinology Study Section of the National Institutes of Health.

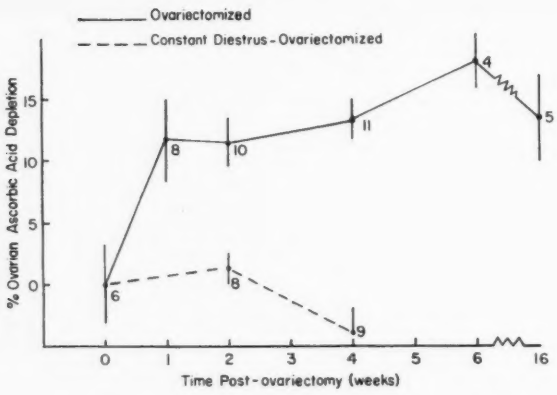

FIG. 1. Plasma LH after ovariectomy of normal and constant-diestrus rats. The vertical lines indicate the standard error of the mean; the numbers refer to the number of assay rats used.

($P<0.01$) on comparison with the value obtained from a pool of normal glands assayed simultaneously.

In rats with CE, pituitary LH was significantly diminished (Table 1), but, as in the rats without lesions, a highly significant increase ($P<.01$) in LH content was seen one month postovariectomy. Assay of pituitary glands from rats with CD gave a very low value for LH content, only 15% of that found in normal rats (Table 1). This value was also significantly lower than that obtained in the rats with CE. In contrast to the observations in normal and CE rats, ovariectomy was not followed by a significant elevation of pituitary LH in these rats with CD.

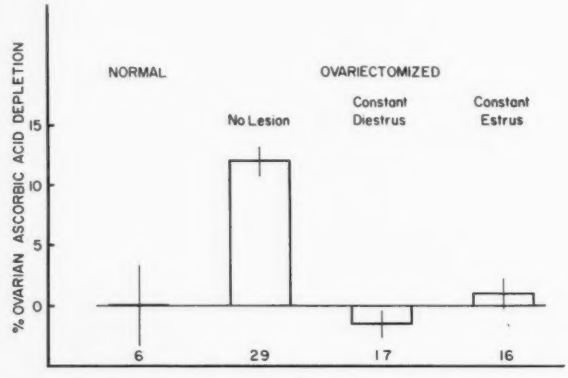

FIG. 2. Plasma LH in normal rats, ovariectomized rats and ovariectomized rats wit lesions inducing either constant diestrus or constant estrus. The vertical lines indicat the standard error of the mean; the numbers refer to the number of assay rats used.

TABLE 1. PITUITARY LH IN RATS WITH HYPOTHALAMIC LESIONS

Type of rat	Assay No.	Pituitary LH	
		Content (μg./gland)	Concentration (μg./mg. wet wt.)
Normal	1	22.0 (9.9–37)[1]	
	2	14.6 (7.5–28)	1.6 (0.76–3.1)
	3	25.6 (13.1–49)	
	4	29.8 (14.5–61)	2.4 (1.2–5.0)
Unweighted Mean		22.2 (16.5–29.9)	2.0 (1.2–3.3)
Ovariectomized	1	36.8 (20.4–66)	4.1 (2.3–7.6)
Constant Estrus	1	8.8 (4.3–17.9)	0.54 (.27–1.1)
	2	5.8 (2.9–11.5)	0.36 (.18–.72)
Unweighted Mean		7.2 (4.4–11.2)	0.44 (.27–.69)
Constant Estrus-Ovariectomized	1	21.1 (10.3–43)	2.0 (1.0–4.1)
	2	27.9 (13.2–59)	2.7 (1.3–5.6)
Unweighted Mean		24.3 (14.5–41)	2.3 (1.4–3.9)
Constant Diestrus		3.3 (1.7–6.7)	0.46 (.23–.92)
Constant Diestrus-Ovariectomized		4.5 (2.3–8.8)	0.83 (.42–1.6)

[1] Figures in parenthesis represent 95% confidence limits.

Pituitary Weight and LH Concentration. A significant atrophy of the anterior lobe was found in animals with hypothalamic lesions inducing CD, whereas those with CE had elevated pituitary weights (Table 2). Pituitary weight fell to normal levels after ovariectomy of the rats with CE, whereas ovariectomy in normal rats and those with CD had no significant effect on the weight of the gland.

These fluctuations in pituitary weight influenced the values for pituitary LH concentration (Table 1). For example, the pituitary LH content of the CE rats was significantly greater than that found in the animals with CD

TABLE 2. ORGAN AND BODY WEIGHTS OF RATS WITH HYPOTHALAMIC LESIONS

Type of rat	Organ weights					Body weight (g.)
	Anterior pituitary (mg.)	Ovaries (mg.)	Uterus (mg.)	Adrenals (mg.)	Thyroid (mg.)	
Normals:						
A. Estrous Smear (5)[1]	11.6±0.9[2]	61±6	428±36	54±4	13.3±1.1	216
B. Diestrous Smears (5)	13.1±0.5	97±10	348±31	67±2	14.0±1.3	275
Pooled Normals (10)	12.3±0.6	79	388	60±3	13.6±0.8	245
Lesions:						
A. Constant Estrus (5)	16.2±1.1*	36±3**	556±41*	52±4	16.9±1.4[3]	290
B. Constant Diestrus (5)	7.3±1.3**	30±6**	185±46*	37±12*	13.3±1.3	288
Lesions-Ovariectomized:						
A. Constant Estrus (8)	10.5±0.9[4]	30±1**[5]				342
B. Constant Diestrus (5)	5.4±8**	39±4**[5]				301

[1] No. of rats/group.
[2] Mean ± S.E.M.
[3] Significantly different from pooled normals, $P < 0.05$.
[4] Significantly different from non-ovariectomized rats with CE, $P < 0.01$.
* Significantly different from appropriate control group, either estrus or diestrus normals as the case may be. $P < 0.05$.
** Significantly different from appropriate control group, $P < 0.01$.
[5] Weight of ovaries at time of ovariectomy.

whereas pituitary LH concentration was identical in both CE and CD rats. This was due to the presence of enlarged pituitaries in the rats with CE, whereas those with CD had atrophic glands. Similarly, in the CE rats, ovariectomy resulted in a more marked increase in pituitary LH concentration than in LH content since a rise in content was associated with a decline in pituitary weight. In other comparisons the direction and approximate relative magnitude of changes in pituitary LH were the same whether calculated as content or concentration.

Organ Weights. Body weights and weights of a group of endocrine organs and the uteri from a series of normals and the rats with lesions are set forth in Table 2. The greater weights of the rats with lesions as compared to normals was due to a large extent to the development of obesity in some of the former animals. There was marked ovarian atrophy in both groups of rats with lesions, while the uteri were atrophic in rats with CD and slightly enlarged in those with CE. Some of the rats with CD also had adrenal atrophy, whereas thyroid weights were unchanged except in rats with CE where a slight increase in weight of this gland was noted.

Histological Observations

Location of Lesions. The lesions in the CD rats were in the median eminence and were similar to those earlier reported (14). Essentially, a complete or nearly complete destruction of this structure was achieved extending back to and frequently including the hypophysial stalk. There was extensive damage to the arcuate nuclei as well. The pituitary vasculature frequently appeared to be normal, but in some cases unilateral or bilateral scars characteristic of old pituitary infarcts were seen.

In rats with CE, on the other hand, the lesions involved the mid-line suprachiasmatic region producing injury or destruction of the suprachiasmatic nuclei and optic chiasm. The lesions frequently extended caudally in the basal hypothalamus so as to reach the anterior tip of the median eminence. This rostral tip was occasionally involved in the lesions. The paraventricular nuclei were usually, though not always, severely injured or destroyed. Because of the rostral location of lesions, there was little or no damage to the hypophysial portal vessels in this type of lesion, and no vascular damage to the pituitary was detectable.

Ovaries. The ovaries of the CE rats were characterized by abundant, large, frequently cystic follicles, abundant interstitium and a paucity of corpora lutea or their remnants (Fig. 3). In contrast, ovaries from CD rats (particularly if operated upon in estrus) were characterized by persistence of large corpora lutea and little or no follicular development (Fig. 3); occasionally one or two follicles which had matured beyond the early antral stage were found. Interstitial tissue in these ovaries was scanty.

Mammary Glands. These were examined grossly and microscopically in some of the rats with lesions, two to three months postoperatively

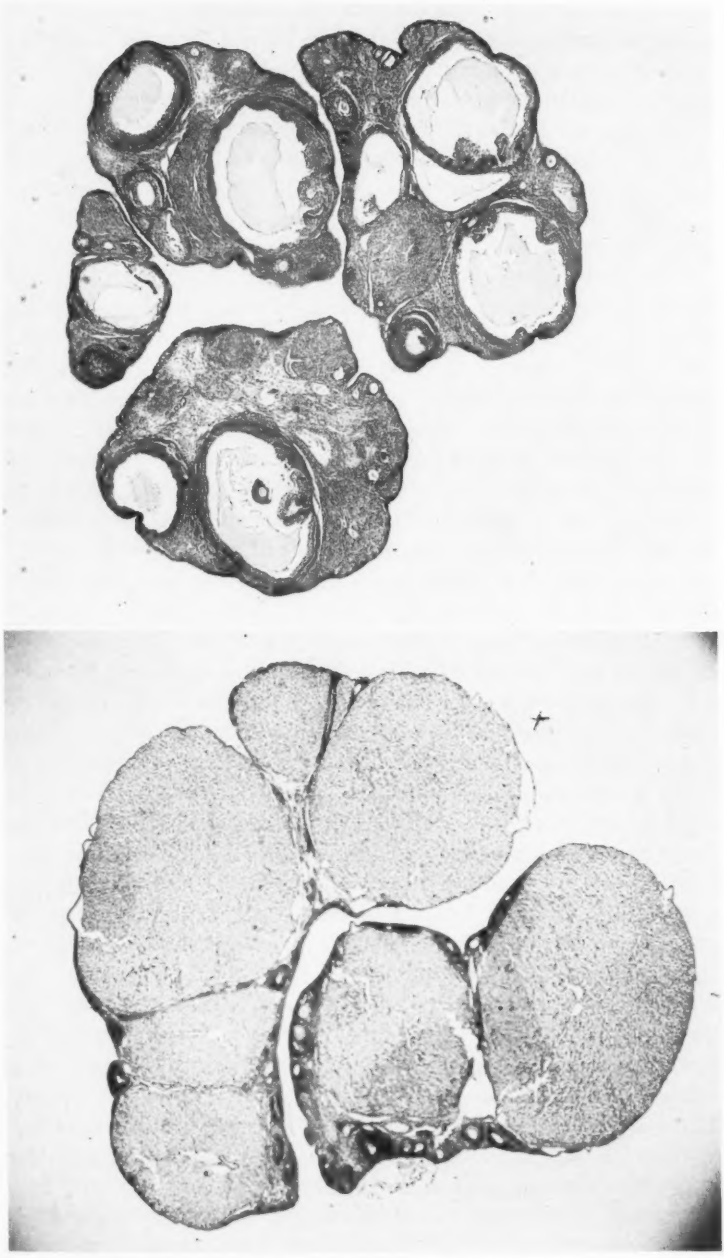

FIG. 3. Sections at the same magnification of ovaries of rats with lesions, on the top from a rat with constant estrus and on the bottom from a rat with constant diestrus.

Mammae from four of five rats with CD showed some evidence of lobuloalveolar development which varied from a barely detectable amount to a quantity of parenchyma nearly as great as that found in pseudopregnancy. There was secretion in the ducts and alveoli. Three mammae from rats with CE were examined microscopically and showed ductile growth and minimal to moderate lobuloalveolar development.

DISCUSSION

Ovarian ascorbic acid depletion has been shown to be a specific assay for LH since other anterior lobe hormones do not induce comparable depletion (10, 11). Vasopressin does have appreciable activity in this assay but only at pharmacological doses which do not interfere under the present conditions (10, 11).

This method is not sufficiently sensitive to measure LH in the plasma of normal rats, but detectable levels of circulating LH are reached within a week following ovariectomy and are maintained for several months. Pituitary LH stores are also elevated following ovariectomy, indicating that removal of negative steroid feedback from the ovaries results in enhanced rates of synthesis and secretion of LH by the adenohypophysis. It has long been known that ovariectomy results in elevated levels of gonadotrophins in blood (15), and similar findings with respect to LH have already been reported by Parlow (16).

Although it is well known that hypothalamic lesions can alter ovarian function, the present work shows the effect of these lesions on LH for the first time. At least two kinds of hypothalamic lesions are able to modify adenohypophysial activity in relation to this hormone. Both the suprachiasmatic lesions and those in the median eminence prevented the increased blood concentrations of LH following ovariectomy. Pituitary LH stores were also subnormal in rats with lesions, which in the presence of reduced blood levels after ovariectomy implys reduced rates of both synthesis and secretion of LH in these rats. In the case of the CD rats, pituitary LH failed to rise after ovariectomy suggesting that both the increased synthesis and release of hormone which follows removal of ovarian steroid feedback failed to occur in these rats. In contrast, the CE rats, although failing to show detectable quantities of hormone in blood, nonetheless manifested an increased storage of LH after ovariectomy implying that synthesis of LH was augmented. The reason for this discrepancy between the behavior of the CE and CD rats is not apparent, but it appears that the lesions in the CE rat produce only a partial blockage of LH release and synthesis. In order to make a rigorous interpretation of the LH data, it would be necessary to know the blood level of LH in rats with intact ovaries and to know the rates of disappearance of LH from blood and its rate of degradation in the hypophysis in all groups of animals.

It is important to inquire as to the mechanism by which these lesions in

fluence LH synthesis and release. In rats with median eminence lesions, the reduction in pituitary weight and frequent infarcts found in this gland raise the possibility that these results may have been influenced by vascular damage to the pars distalis caused by interruption of the hypophysial portal veins. The recent demonstration of Nikitovitch-Winer and Everett that pituitaries regrafted to the median eminence can regain function in spite of severe vascular insult and reduced volume argues against this possibility (17). Rather, it sould seem more likely that the lesions have interfered with the secretion of an LH-releasing factor which may normally traverse the hypophysial portal vessels to induce LH secretion.

The suprachiasmatic lesions did not affect the hypophysial vasculature, so that the deficit in these rats is almost certainly a specific one due to loss of hypothalamic influences. It is possible that this suprachiasmatic region may be a chemoreceptor zone for ovarian steroids, as suggested by the ability of ovarian grafts in this region to decrease uterine weight (18). From it, axons may project to the median eminence-arcuate nuclear region to affect neurons secreting an LH-releasing factor. Alternatively, it is conceivable that the neurosecretory cells themselves might lie in this rostral area and that their axons might project to the median eminence, there to secrete LH-releasing factor. Further work is required to determine the actual mechanism.

Previous work by Bogdanove in male rats has indicated that hypothalamic lesions associated with testicular atrophy (or even on occasion without gonadal atrophy) are accompanied by low hypophysial stores of gonadotrophin (19). No assays for LH were reported. Recently, Davidson, *et al.* have found testicular atrophy and low pituitary LH content in dogs with lesions in the caudal median eminence (20). Our results in the female rat are in general agreement with these reports in the male. In our experiments in rats with median eminence lesions, however, the caudal median eminence was frequently intact. Thus it is not yet possible to ascribe a particular importance to this area for regulation of LH secretion in the rat.

That the suprachiasmatic area and rostral median eminence may be important in regulation of LH secretion is further supported by the experiments of Critchlow, who has recently produced ovulation from stimulation of these portions of the hypothalamus in the rat (21).

The changes recorded in organ weights in rats with lesions can be correlated with altered secretion rates of pituitary and ovarian hormones. Decreased ovarian weights were seen in both types of rats with lesions. In the case of the CD rats, the decrease was accounted for by the small size of follicles and scanty interstitial tissue presumably caused by deficient secretion of FSH and LH, respectively (9). In the case of the CE rat, the decreased ovarian weight was presumably the result of the marked decrease in luteal tissue, probably related to the failure of ovulation. The uterine atrophy found in the CD rats, probably followed the decreased estrogen

secretion as a consequence of decreased FSH-LH output, whereas the uterine hypertrophy in the CE animals reflected the continuous estrogen secretion as evidenced by persistent vaginal cornification. Enlargement of the pituitary in rats with CE is reminiscent of the increased size of this gland which can be produced by estrogen treatment (22). Since persistent estrogen secretion occurred in these rats, and the pituitary weights were normal after ovariectomy, it seems likely that estrogen was responsible for the effect. D'Angelo has also reported enlargement of the pituitary in rats with CE (23).

The mammary development seen in the rats with both types of hypothalamic lesions is consistent with persistent secretion of LTH and suggests that the secretion of this hormone noted earlier in rats with median eminence lesions (2) can persist for several months just as it does when the pituitary is grafted to the kidney capsule (24). There appears to be a reciprocal relationship between the secretion of LTH and LH by the isolated pituitary.

REFERENCES

1. HARRIS, G. W.: in *Handbook of Physiology II*, Amer. Physiol. Soc., Washington, 1960, p. 1007.
2. MCCANN, S. M. AND H. M. FRIEDMAN: *Endocrinology*. In press.
3. HILLARP, N. A.: *Acta Endocrinol.* **2:** 11. 1949.
4. GREER, M. A.: *Endocrinology* **53:** 390. 1953.
5. ALLOITEAU, J. J.: *Compte Rendu Soc. Biol.* **148:** 223. 1954.
6. VAN DYKE, D. C., M. E. SIMPSON, S. LEPKOVSKY, A. A. KONEFF AND J. R. BROBECK: *Proc. Soc. Exper. Biol. and Med.* **95:** 1. 1957.
7. D'ANGELO, S. A. AND A. KRAVATZ: *Anat. Rec.* **133:** 370. 1959.
8. GALE, C.: Doctoral Dissertation, University of Pennsylvania, 1960.
9. EVANS, H. M. AND M. E. SIMPSON: in *The Hormones II*, ed. by Pincus, G. and K. V. Thiman, Academic Press, New York, 1950, p. 351.
10. PARLOW, A. F.: *Fed. Proc.* **17:** 402. 1958.
11. MCCANN, S. M., S. TALEISNIK AND H. FRIEDMAN: *Fed. Proc.* **19:** 292. 1960.
12. MINDLIN, R. L. AND A. M. BUTLER: *J. Biol. Chem.* **122:** 673. 1937.
13. GOMORI, G.: *Am. J. Path.* **17:** 398. 1941.
14. MCCANN, S. M., R. MACK AND C. GALE: *Endocrinology* **64:** 870. 1959.
15. FLUHMAN, C. F.: *Am. J. Obstet. and Gyn.* **20:** 1. 1930.
16. PARLOW, A. F.: *Program of the 41st Meeting of the Endocrine Society*, p. 46, 1959.
17. NIKITOVITCH-WINER, M. AND J. W. EVERETT: *Endocrinology* **65:** 357. 1959.
18. FLERKO, B. AND J. SZENTAGOTHAI: *Acta Endocrin.* **26:** 121. 1957.
19. BOGDANOVE, E. M., B. N. SPIRTOS AND N. S. HALMI: *Endocrinology* **57:** 302. 1955.
20. DAVIDSON, J. M., A. N. CONTOPOULOS AND W. F. GANONG: *Endocrinology* **66:** 735. 1960.
21. CRITCHLOW, B. V.: *Am. J. Physiol.* **195:** 171. 1958.
22. MCEUEN, C. S., H. SELYE AND J. B. COLLIP: *Lancet* **1:** 775. 1936.
23. D'ANGELO, S. A. AND J. SNYDER: *Fed. Proc.* **19:** 272. 1960.
24. NIKITOVITCH-WINER, M. AND J. W. EVERETT: *Endocrinology* **62:** 522. 1958.

PURIFICATION AND PROPERTIES OF A COMPONENT OF THE PITUITARY GLAND WHICH PRODUCES LIPEMIA IN THE RABBIT[1]

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ABSTRACT

A modification of the previously described method for the preparation of Fraction H from hog pituitary glands by alkaline extraction, fractional precipitation with acetone, and chromatography on IRC-50 resin is presented. The yield is 8 mg. per gram of lyophilized hog pituitary glands. As little as 0.125 mg. of Fraction H causes a 6 fold rise in the serum free fatty acid level, and as little as 3 mg. causes a 4 fold increase in the serum total lipid level of the rabbit. Fraction H prepared by this method contains 0.8% growth hormone, 0.007% oxytocin, 0.00005% melanocyte-stimulating hormone, and no detectable amount of the other 6 recognized pituitary hormones. The active substance in Fraction H is inactivated by trypsin or by exposure to 0.1N NaOH at 100° C, is non-dialyzable, and is insoluble in 5% trichloracetic acid. The biologic activity of Fraction H is not diminished by digestion with pepsin or by exposure to 0.1N HCl at 100° C. The active substance or substances in the peptic digest of Fraction H are dialyzable and are soluble in 5% trichloracetic acid. Chromatography of Fraction H on DEAE-cellulose gives a further 5-fold purification of the active component. The resulting preparation (labeled Fraction L) moves as a single substance in paper electrophoresis.

A RECENT report (1) from this laboratory described the preparation from hog pituitary glands of a fraction (labeled "Fraction H") which produces lipemia in the rabbit. Bioassays showed this fraction to contain 0.07% TSH,[3] 0.002% oxytocin, and no detectable amount of ACTH, GH, prolactin, FSH, ICSH, or vasopressin. A subsequent study (2) showed that

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[2] Senior Research Fellow of the New York Heart Association 1957–1960. New York City Investigator, Contract I-118.

[3] The following abbreviations are employed: ACTH, adrenocorticotropin; GH, growth hormone; TSH, thyroid-stimulating hormone; ICSH, interstitial-cell stimulating hormone; FSH, follicle-stimulating hormone; MSH, melanocyte-stimulating hormone.

the immediate effect of an injection of Fraction H in the rabbit is a marked increase in the concentration of serum free fatty acids (FFA), which is followed 4 to 8 hours later by the development of lipemia.

The purpose of the present report is to describe a method for preparing Fraction H from hog pituitary glands in higher yield, certain physical and chemical properties of the biologically active substance in Fraction H, and a method for the further purification of this substance.

MATERIALS AND METHODS

1. Lyophilized intact hog pituitary glands (Armour Pharmaceutical Company).

2. Cation exchange resin IRC-50 (Rohm and Haas Company, CG-50 Type 2) equilibrated with 0.2M sodium phosphate buffer pH 5.80. 250 gm. of the resin were suspended in 5 L. 0.2M sodium phosphate buffer with continuous stirring. Forty per cent NaOH was added periodically until the pH of the suspension was stable at 5.80 ± 0.1. The resin was then washed by decantation with 0.2M sodium phosphate buffer pH 5.80 until the supernatant was clear after standing for 30 minutes. The washed resin was stored under the 0.2M buffer until needed.

3. Diethylaminoethyl cellulose anion exchange resin (DEAE-cellulose) (Distillation Products Industries) was prepared for use by suspending it in 20 volumes of 0.01M sodium phosphate buffer pH 5.40, adjusting the pH to 5.9 with HCl and then washing by decantation with 0.01M sodium phosphate buffer pH 5.40 until the pH remained constant at 5.40.

4. Recrystallized pepsin and trypsin (Nutritional Biochemical Company).

5. Male rabbits of mixed strain weighing 3.5 to 4.5 kg. were employed. They were fed a diet of Purina rabbit chow which was available to the animals at all times. The lipid-mobilizing activity of the various pituitary preparations described in this report was estimated by measuring the effect of a single subcutaneous injection of an aqueous solution of the preparation upon the concentration of total lipid (1) or of FFA (2) in the serum of the rabbit. Serum concentrations of FFA and total lipid were measured by the methods of Dole and of Bloor respectively (3, 4). Other pituitary hormonal activities were assayed by methods described in the following references: ACTH (5), GH (6), TSH (7), prolactin (8), ICSH (9), FSH (10), MSH (11), oxytocin and vasopressin (12).

EXPERIMENTS

Certain modifications in the previously described method (1) for the preparation of Fraction H led to an increased yield of material. These modifications are as follows: After extraction of the pulverized glands for 4 hours with 2% NaCl at pH 8.5, the extract was adjusted to pH 4.5 instead of 4.3 as previously described. After removal of the material precipitated at this point, 2 volumes of acetone were added and the precipitate was discarded. The concentration of acetone was brought to 90% and the precipitated material isolated by solution in H_2O at pH 5.80, followed by dialysis and lyophilization. Previously the fraction soluble in 75% acetone and insoluble in 90% acetone was isolated at this point. The yield of this fraction ("Fraction G") was twice that obtained previously. Fraction G in batches of 80 mg. instead of 120 mg. was chromatographed as previously described on a 2.2×30 cm. column of IRC-50 resin equilibrated with 0.2M sodium pho-

TABLE 1. RESULTS OF BIOASSAYS OF FRACTION H

Left-hand column shows assays performed. Middle column gives result of each assay. Right-hand column gives proportion by weight in which each pituitary hormone is present in Fraction H. These proportions were calculated by comparing hormonal activities of Fraction H with activities of highly purified preparations of each of the pituitary hormones.

Hormonal activity assayed	Hormonal activity of Fraction H	Proportion in which each pituitary hormone is present in Fraction H (%)
ACTH	<0.04 unit/mg.	<0.03
GH	0.01 unit/mg.	0.8
TSH	<0.001 unit/mg.	<0.007
Prolactin	<0.002 unit/mg.	<0.01
ICSH	No activity detected at total dose of 5 mg.	<0.4
FSH	No activity detected at total dose of 2.5 mg.	<0.06
MSH	7.5×10^5 units/gm.	0.00005
Oxytocin	0.04 unit/mg.	0.007
Vasopressin	<0.004 unit/mg.	<0.002

phate buffer pH 5.80. The yield of Fraction H was increased from 5 mg. to 8 mg. per gram of lyophilized gland by these modifications in the procedure.

These modifications did not change the potency of Fraction H in stimulating the mobilization of FFA and total lipid into the plasma. Bioassays[4] for the 9 recognized hormonal activities of the pituitary gland showed that Fraction H prepared by this method contained 0.8% GH, 0.007% oxytocin, 0.00005% MSH, and no detectable amount of any of the other 6 recognized pituitary hormones. Details of these assays are shown in Table 1.

Further purification of the active component in Fraction H was achieved by chromatography of this fraction on DEAE-cellulose. Three mg. of Fraction H was dissolved in 4 ml. of 0.01M sodium phosphate buffer (pH 5.40) and applied to a 0.9×15 cm. column of DEAE-cellulose which had been equilibrated to pH 5.40 by washing with this buffer. Elution was started with 0.01M sodium phosphate buffer (pH 5.40), the effluent being collected in 5 ml. fractions and the protein content of each fraction estimated from the optical density at 278 mμ. After 35 ml. of the 0.01M sodium phosphate solution had passed into the column, elution was continued successively with 35 ml. each of 0.05 M, 0.15 M, and 0.30 M sodium phosphate solutions of pH 5.40, pH 5.50 and pH 5.60 respectively. The results are shown in Figure 2. Only the fractions eluted from the column by the 0.15 M solution (tubes 15 through 25 in Fig. 1) possessed activity in raising the

[4] Assays were performed by the following: Duplicate ACTH assays by J. D. Fisher (Armour Pharmaceutical Co.) and C. N. Mangieri (South Mountain Laboratories, Maplewood, N. J.); GH and prolactin assays by C. N. Mangieri; TSH assay by R. W. Bates (National Institutes of Health); ICSH assay by S. J. Segal (Rockefeller Institute); FSH assay by S. L. Steelman (Merck Institute); MSH assay by A. B. Lerner (Yale University); oxytocin and vasopressin assays by F. C. Armstrong (Parke, Davis & Co.). The authors are grateful for the collaboration of these investigators.

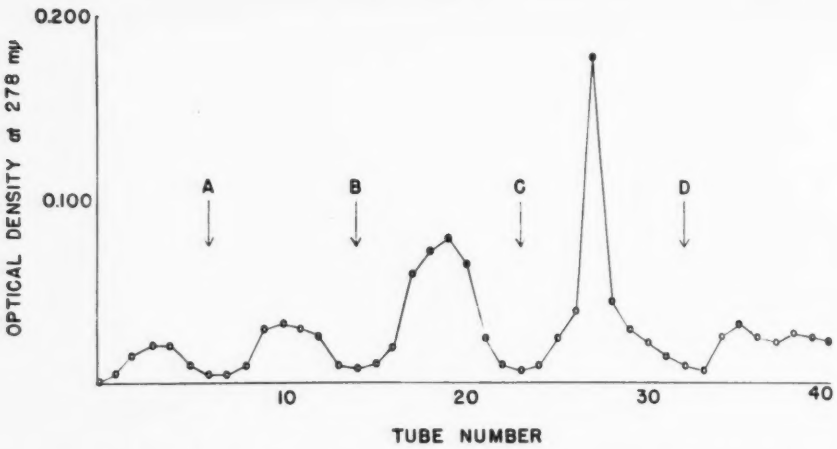

FIG. 1. Chromatogram of 3 mg. Fraction H on 0.9×15 cm. column of DEAE-cellulose. Effluent collected in 5 ml. fractions. Initial solvent, 0.01 M sodium phosphate buffer, pH 5.40. At A, solvent changed to 0.05 M sodium phosphate buffer, pH 5.40; at B, to 0.15 M sodium phosphate buffer, pH 5.50; at C, to 0.30 M sodium phosphate buffer, pH 5.60; at D, to 1.0 N HCl. Flow rate, 20 ml./hour. Temperature 25° C.

concentrations of FFA and total lipid in the rabbit's serum. A sample of this material, which is labelled Fraction L, was prepared by chromatography of 15 mg. of Fraction H on a 0.9×30 cm. column of DEAE-cellulose in the same manner described in Figure 1. The fractions eluted by the 0.15 M sodium phosphate buffer were combined and 0.42 gm. of $(NH_4)_2SO_4$ was added for each ml. of eluate. The resulting precipitate was dissolved in H_2O, dialyzed and lyophilized. Three mg. of Fraction L was obtained. The effect of Fraction L upon the serum FFA and total lipid concentrations of adult unfasted rabbits is shown in Table 2 together with comparable data obtained with Fraction H. It can be seen that although the yield of Fraction L obtained from Fraction H was only 20%, its activity was 5 times as great as that of Fraction H. No activity was lost and a 5

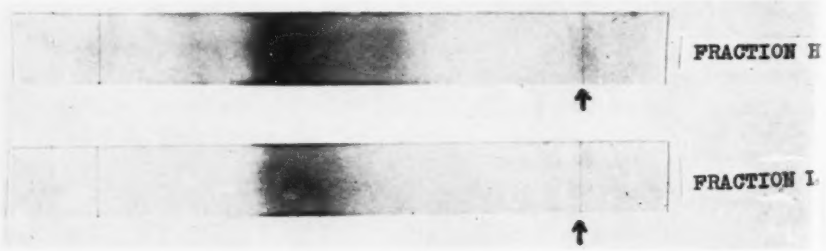

FIG. 2. Paper electrophoresis of Fraction H and of Fraction L at pH 8.6 in 0.075 M veronal buffer. Arrow indicates the origin.

TABLE 2. EFFECT OF SUBCUTANEOUS INJECTION OF VARIOUS DOSES OF FRACTION H AND OF FRACTION L UPON THE CONCENTRATION OF FFA (1 HOUR AFTER INJECTION) AND OF TOTAL LIPID (10 HOURS AFTER INJECTION) IN THE RABBIT'S SERUM

Material injected	Serum FFA concentration (ueq./L) 1 hour after injection	Serum total lipid concentration (mg. %) 10 hours after injection
H_2O	257 ± 24 (5)*	360 ± 150 (5)
0.0625 mg. Fraction H	310 ± 58 (4)	380 ± 130 (3)
0.125 mg. Fraction H	1490 ± 290 (4)	—
0.25 mg. Fraction H	2510 ± 374 (4)	—
0.50 mg. Fraction H	2650 ± 351 (4)	—
1.0 mg. Fraction H	2840 ± 265 (4)	—
3.0 mg. Fraction H	2410 ± 340 (4)	1590 ± 310 (4)
0.0125 mg. Fraction L	290 ± 76 (3)	—
0.025 mg. Fraction L	1512 ± 383 (3)	—
0.05 mg. Fraction L	2420 ± 424 (3)	—
0.1 mg. Fraction L	2010 ± 603 (3)	—
0.6 mg. Fraction L	2440 ± 184 (4)	1480 ± 430 (4)

* Average ± Standard Error (number of observations).

fold purification had been accomplished. Only one protein component was detectable in Fraction L by paper electrophoresis (Fig. 2). The rate of migration of Fraction L was found to be 0.8× that of human serum albumin at pH 8.6.

The capacity of the DEAE-cellulose column described above is so limited that difficulties have been encountered in preparing sufficient quantities of Fraction L for a study of its chemical and physical properties. As the presence of the inert substances in Fraction H have no apparent effect upon the activity of the active component of this fraction, these studies have been carried out on this more available fraction.

(1) *Effect of heating in acid or alkali.* 3.0 mg. of Fraction H was dissolved in 6 ml. of 0.1N HCl, placed in a boiling water bath for 30 minutes, cooled, neutralized and made up to a volume of 12 ml. The injection of 2 ml. of this solution (containing 0.5 mg. of treated Fraction H) in each of 4 rabbits caused a rise in serum FFA level 1 hour after the injection to an average level of 2600 μeq./L. (Table 3). In contrast, treatment for 15 minutes at 100° C with 0.1N NaOH resulted in a complete loss of the activity of Fraction H (Table 3).

(2) *Effect of digestion with pepsin or trypsin.* 3.0 mg. of Fraction H in 0.01N HCl was incubated for 1 hour at 25° C with 0.075 mg. of crystalline pepsin. One sixth of the volume of the neutralized incubation mixture (containing 0.5 mg. of treated Fraction H) was injected in each of 4 rabbits. An increase in average serum FFA level at 1 hour to 3194 μeq./L. was produced (Table 3). Similar results were obtained with digestion periods as long as 17 hours. In contrast, incubation with crystalline trypsin (1 part of trypsin to 40 parts of Fraction H) at pH 9.0 for as short a period as 1 hour resulted in a complete loss of activity (Table 3). The disappearance of the

biologic activity of Fraction H following digestion with trypsin indicated the presence of a peptide chain in the active component, while the endurance of the biologica activity following digestion by pepsin suggested that the activity might reside in a limited pepsin-resistant portion of the peptide chain.

(3) *Solubility in 5% trichloracetic acid (TCA).* TCA solution (50%) was added to 3.0 mg. of Fraction H in 10 ml. of H_2O at 0° C to a final concentration of 5% TCA. The precipitate was collected by centrifugation at 0° C and dissolved in H_2O. The supernatant was extracted 4 times with an equal volume of ethyl ether to remove the TCA. The solution of the 5% TCA precipitate, and the ether-extracted 5% TCA supernate, were then tested for their effect on the rabbit's serum FFA level at dosages representing $\frac{1}{6}$ of the total precipitate and $\frac{1}{6}$ of the total supernate. The 5% TCA precipitate of Fraction H was highly active in increasing the serum FFA level, while the 5% TCA supernate was inactive (Table 3). In contrast, when 3.0 mg. of Fraction H was incubated for 3 to 17 hours with 1/40 part of pepsin prior to TCA precipitation, the 5% TCA precipitate was inactive and the 5% TCA supernate was highly active in increasing the rabbit's serum FFA concentration (Table 3). These findings suggested that the active substance in Fraction H, which is insoluble in 5% TCA, is hydrolyzed by pepsin with the release of one or more smaller active components which are soluble in 5% TCA.

(4) *Dialyzability.* Three mg. of Fraction H, or 0.6 mg. of Fraction L, dissolved in 5 ml. of 0.01 M, pH 7.0 sodium phosphate buffer, was dialyzed for 8 hours at 5° C with continuous stirring against 100 ml. of the buffer. At the end of the dialysis period, the inner and outer solutions (containing

TABLE 3. EFFECT UPON BIOLOGIC ACTIVITY OF FRACTION H OF EXPOSURE TO HEAT, ENZYMES, OR TRICHLORACETIC ACID (TCA)

Material injected†	Serum FFA level 1 hour after injection (μeq./L)
H_2O	257 ± 26*
Fraction H after 1 hour digestion with pepsin**	3194 ± 365
Fraction H after 17 hour digestion with pepsin**	3533 ± 118
Fraction H after 1 hour digestion with trypsin**	273 ± 5
Fraction H after 30 min. at 100° C in 0.1N HCl	2600 ± 600
Fraction H after 15 min. at 100° C in 0.1N NaOH	213 ± 10
5% TCA precipitate of Fraction H	1954 ± 72
5% TCA supernate of Fraction H	327 ± 87
5% TCA precipitate of peptic digest†† of Fraction H	214 ± 21
5% TCA supernate of peptic digest†† of Fraction H	2820 ± 430

* Average ± standard error in a group of 4 rabbits.

† The starting material in each experiment was 3.0 mg. of Fraction H. Following treatment with pepsin, trypsin, HCl, NaOH, TCA, or pepsin followed by TCA, the resulting preparations were tested in a group of 4 rabbits at a dose representing $\frac{1}{6}$ of the amount of the preparation.

** 1 part of enzyme to 40 parts of Fraction H.

†† Digestion with pepsin (1/40) for 17 hours.

TABLE 4. BEHAVIOR OF ACTIVE COMPONENT IN FRACTION H, IN FRACTION L AND IN PEPTIC DIGEST OF FRACTION H DURING 8 HOUR DIALYSIS

Preparation	Serum FFA level 1 hour after injection (μeq./L)	
	Inner solution* (Non-dialyzable material)	Outer solution* (Dialyzable material)
3.0 mg. Fraction H (at pH 7.0)	3066 ± 132†	225 ± 32
3.0 mg. Fraction H (in 2.0N Acetic Acid)	1200 ± 92	361 ± 35
0.6 mg. Fraction L (at pH 7.0)	2087 ± 432	265 ± 15
5% TCA supernate of peptic digest of 3.0 mg. of Fraction H (at pH 7.0)	3709 ± 387	2818 ± 400

* Each solution was tested in 4 rabbits at a dose representing $\frac{1}{6}$ of the volume of the solution.

† Average ± standard error for a group of 4 rabbits

the non-dialyzable and dialyzable materials respectively) were tested for effect on the rabbit's serum FFA level at dosages representing $\frac{1}{6}$ of the volume of each solution. Table 4 shows that no detectable activity appeared in the outer solution of the dialysis system, suggesting that the active substance in Fraction H and in Fraction L is either a protein or a peptide tightly bound to protein. Since certain hormones which occur as peptide-protein complexes have been found to dissociate in acetic acid (13), the dialyzability of the active substance in Fraction H was re-examined in 2N acetic acid. At the end of the 8 hour dialysis period, both inner and outer solutions were lyophilized to remove the acetic acid and the residues were dissolved in H_2O for assay. Again no activity was detected in the dialyzate (Table 4). The diminished activity of the non-dialyzable material in this experiment (Table 4) may have been caused by partial inactivation during the 8 hour exposure to 2N acetic acid and subsequent lyophilization, or by binding of the active substance to the dialysis membrane.

The dialyzability of the active substances in the peptic digest of Fraction H was now examined. Three mg. of Fraction H was incubated with 0.075 mg. of pepsin in 0.01N HCl for 17 hours. After precipitation of the peptic digest with TCA, the 5% TCA supernatant was extracted with ether, neutralized, and dialyzed for 8 hours against 20 volumes of pH 7.0 sodium phosphate buffer. Table 4 shows that both the inner and outer solutions of the dialysis system were highly active in increasing the rabbit's serum FFA level. This finding supports the hypothesis that one or more of the active substances in the peptic digest of Fraction H are of relatively low molecular weight.

DISCUSSION

Modification of the acetone fractionation step in the previously described method for preparing Fraction H has led to a 60% increase in the

yield of this material without reduction in the lipid-mobilizing potency. Fraction L, which is obtained from Fraction H by chromatography on DEAE-cellulose, constitutes $\frac{1}{5}$ of the weight of Fraction H and possesses about 5× the lipid-mobilizing potency of Fraction H. Only one protein component is detectable in Fraction L by electrophoretic analysis. An amount of Fraction L sufficient for further studies of its state of purity has not yet been prepared.

The biologic activity of Fraction H is rapidly destroyed by exposure to trypsin or to 0.1N NaOH at 100° C. No biologic activity could be detected in the dialyzate of Fraction H or of Fraction L. The active substance in Fraction H is insoluble in 5% TCA. These findings suggest that the active substance in Fraction H contains peptide bonds and either is a protein or is a peptide tightly bound to protein or other non-dialyzable material.

The endurance of the biologic activity following incubation with pepsin or exposure to 0.1N HCl at 100° C suggests that the activity is a function of only a limited portion of the peptide chain of the active substance. Following digestion of Fraction H with pepsin, the active substance or substances are completely soluble in 5% TCA and pass through the dialysis membrane. These observations suggest that pepsin cleaves the non-dialyzable active substance in Fraction H with release of a dialyzable, biologically-active fragment of the naturally occurring substance. An alternative possibility is that the active substance in Fraction H and in Fraction L is a dialyzable peptide tightly bound to protein, and that the apparent change in properties of the active substance following exposure to pepsin results from hydrolysis of the inert protein with release of the naturally occurring active peptide from a peptide-protein complex.

REFERENCES

1. Rudman, D., F. Seidman and M. B. Reid: *Proc. Soc. Exper. Biol. Med.* **103:** 315. 1960.
2. Di Girolamo, M., D. Rudman, M. B. Reid and F. Seidman: *Endocrinology.* In press.
3. Dole, V. P.: *J. Clin. Invest.* **35:** 150. 1956.
4. Bloor, W. R.: *J. Biol. Chem.* **77:** 53. 1928.
5. U. S. Pharmacopeia, XV revision: 176. 1955.
6. Marx, W., M. E. Simpson and H. M. Evans: *Endocrinology* **30:** 1. 1942.
7. Bates, R. W. and J. Cornfield: *Endocrinology* **60:** 225. 1957.
8. Lyons, W. R. and E. Page: *Proc. Soc. Exper. Biol. Med.* **32:** 1049. 1935.
9. Segal, S. J.: *Science* **126:** 1242. 1957.
10. Steelman, S. L. and F. M. Pohley: *Endocrinology* **53:** 604. 1953.
11. Shizume, K., A. B. Lerner and T. B. Fitzpatrick: *Endocrinology* **54:** 553. 1954.
12. U. S. Pharmacopeia, XV revision: 776. 1955.
13. Dedman, M. L., T. H. Farmer, P. Morris and C. J. D. R. Morris: *Rec. Prog. Hormone Res.* **7:** 59. 1952.

THE COMPOSITION OF RAT UTERINE LUMINAL FLUID

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ABSTRACT

Chemical components of uterine fluid obtained from proestrus and estradiol, estrone and estriol treated ovariectomized rats have been studied. Analyses of fluid from rats with ligated uteri also are presented. Potassium concentrations were ten times greater than plasma values. Activities of β-glucuronidase and alkaline phosphatase in uterine fluids were greater than those observed in plasma. Acid phosphatase was absent from uterine fluid. Protein components having electrophoretic mobilities similar to beta, alpha-2, alpha-1 globulins and albumin of plasma have been identified in luminal fluid. Moreover, uterine fluid contains a protein fraction which has a mobility exceeding that of albumin. These qualitative and quantitative differences support the idea that uterine luminal fluid is an ultrafiltrate of plasma supplemented by secretions from the uterus.

CHARACTERISTIC alterations in the distribution of water in the rat uterus take place during the estrus cycle and such changes can be induced in the castrated animal by the administration of estrogen (1, 2). Long and Evans (1) described the distention of the uterus with fluid in proestrus and early estrus, with discharge occurring during late estrus. Similar accumulation of fluid in the lumen of the uterus has been noted in several species of mammals, although there are considerable differences in relative amounts. These include the mouse (3), bank vole (4), rabbit (5), opossum (6), dog (7), pig (8), monkey (9), and cow (10).

There also appears to be considerable differences among species as to rate of fluid formation in the uterus and factors that influence its formation. DeMarco (7) found that fluid formation in the bitch during heat was increased fourfold by the subcutaneous injection of pilocarpine. A similar though less quantitative observation was reported for the dog by Shih, *et al.* (11). This reaction to pilocarpine also applies to the rabbit oviduct (12). The monkey (Macacus rhesus) seems to be different in that neither pilocarpine nor atropine influences the accumulation of fluid in the uterus, but adrenalin and intravenous saline tend to increase the rate of flow from uteroabdominal fistulae (9).

The problem of accumulation of uterine fluid has also been studied by

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ligating the uterus. Bishop (12) found that fluid did not increase in uteri of rabbits when ligatures were placed at the uterotubal junctions. However, Reynolds and Kaminester (13) observed that in ovariectomized rabbits, fluid accumulated in the lumen of the uterus between two ligatures about one inch apart. Even doubly ligated uterine sacs in castrated rabbits became filled with fluid (13).

Ligation of the uterine cervix of mice (14), rats (15), hamsters (14) and dogs (11) also results in uterine fluid accumulation. By puncturing the uterus at various intervals as much as 100 ml. of fluid has been obtained from a single mouse during a year's time. Castration was found to retard the formation of fluid but not prevent it, and injection of estradiol restored fluid formation (14). Shih, *et al.* (11) obtained from 7 to 17 ml. of fluid from closed uterine segments in the dog.

There also have been several investigations of the biological properties of uterine luminal fluid. Warren (15) studied the effects of injecting uterine fluid into castrated adult female rats. Five daily subcutaneous injections of 2 ml. failed to alter the estrus cycle of castrated animals. Histological examination of reproductive tract, mammary gland and pituitary showed that all tissues were typical of castrated animals. Other experiments considered the effect of removal of uterine fluid on sperm transport.

More recently, Homburger, *et al.* (16) studied various biological actions of uterine fluid collected from ligated uteri of mice. Effects observed following intraperitoneal administration of such fluid to mice include: (1) increased uterine size in castrates; (2) decreased adrenal size; (3) fulminating exophthalmos, retro-ocular hemorrhage and necrotizing ophthalmia.

The partitioning of radioactivity in uterine luminal fluid from rats injected with estrone-16-C^{14} has been reported to result in a recovery in the phenolic fraction of approximately .001% of the total injected radioactivity (17). Bioassay of this fraction by the technique of Astwood (18) proved negative. The only appreciable radioactivity was present in the aqueous residue.

Broome and Lamming (19) have reported that fluid collected from the ligated uteri of oestrus rabbits proved to be excellent media for the growth of *E. coli* and *Staphylococcus aureus.*

Reports have issued on the chemical constituents of uterine fluid from mice (14), rats (11, 20), rabbits (11), dogs (11), pigs (20) and the cow (10). Dissimilarities between rat uterine luminal fluid and plasma have been reported for protein composition (21) and potassium content (20).

The work reported here was undertaken to extend our knowledge of the chemical composition of rat uterine fluid and possibly identify components which would distinguish it from other body fluids.

METHODS

Adult female rats of the Harvard strain (250 gm.) were used throughout this study. Uterine luminal fluid was collected from animals during normal proestrus and

from 7-day postovariectomized rats injected with either estrone (5 μg./day/3 days), estradiol (1 μg./day/3 days) or estriol (0.5 mg./day/3 days). All steroids were dissolved in sesame oil and a volume of 0.2 ml. injected daily. On the fourth day, fluid was withdrawn from the distended uteri by syringe to avert contamination with blood. For most determinations, the pooled fluid from three to five animals was adequate.

Fluid from 80 to 120 estrogen-treated animals was pooled for each electrophoretic determination. Care was taken to maintain the fluid at approximately 5° C. during the extended collection period. The small protein content of uterine fluid required that it be concentrated before analyses. This was accomplished by dialysis in the cold against 20% clinical dextran. The volume and protein concentration of fluid obtained from individual normal adult female rats with ligatures around each branch of the genital tract at the uterotubal junction and at the cervix were sufficient for conducting direct analysis.

Blood was obtained by direct heart puncture with a heparinized syringe, and immediately centrifuged in the cold. The protein concentration of the plasma was adjusted to 1% with appropriate buffer for electrophoretic studies.

Samples of either uterine fluid or plasma, each adjusted with barbiturate buffer, pH 8.6, $r/2 = 0.1$ to approximately 1% protein, were dialyzed with constant stirring against 1 liter of the buffer at 4° C. for 16 hours. Moving boundary electrophoresis was performed in a Perkin-Elmer model 38 instrument using a 2 ml. cell under the following conditions: current: 8.1 ma.; potential gradient: approximately 10.5 V/cm.; temperature: approximately 2.4° C; time: 3,600–7,200 seconds. Migration for each component was measured from the descending pattern photograph and the mobility computed (22). The percentage composition for each component also was calculated.

Total nitrogen determinations were made by nesslerization after digestion in a selenium-sulfuric acid mixture. Samples were dried in the oven at 100–120° C overnight for the calculation of dry weights. Protein was determined by the biuret method of Gornall, *et al.* (23). Sodium and potassium concentrations were estimated from flame photometric measurements. A micro glass electrode was used to determine pH. This measurement was made immediately upon aspiration of the uterine fluid. Analyses also were conducted for phosphorus (24). β-Glucuronidase was quantitated by the method of Talalay, *et al.* (25). Alkaline phosphatase and acid phosphatase were determined by a modification of the method of Schmidt and Thannhauser (26).

Ligated uteri (free of fluid) were fixed in Bouin's solution, washed, imbedded in paraffin, sectioned and stained with hematoxylin and eosin for histological examination.

RESULTS

Uterine luminal fluid is a slightly opalescent, colorless liquid which contains an occasional lymphocyte. On microscopic examination, the only consistent structures noted were black granules. These were not further identified. Shih, *et al.* (11) mentioned an occasional epithelial cell on microscopic examination.

The mean volume of uterine fluid collected during proestrus was 0.37 ml. (Table 1). Estradiol (1 μg./day/3 days) and estrone (5 μg./day/3 days) resulted in similar volumes, while estriol (0.5 mg./day/3 days) resulted in a mean volume approximately twice that observed with estrone or estradiol. Appreciably greater volumes of fluid were obtained from rats with ligated uteri; the amount tending to increase as the period of ligation was extended (Table 1).

Points of similarity in the fluids collected durlng proestrus and estrone or estriol stimulation were the content of water, pH, total nitrogen, protein

TABLE 1. COMPOSITION OF RAT UTERINE LUMINAL FLUID

Measurement	Treatment						
	Proestrus*	Estradiol*	Estrone*	Estriol*	Ligated (days)		
					45†	90†	360†
Volume (ml.)	0.37 (0.1–0.9)	0.24 (0.05–0.7)	0.38 (0.23–0.6)	0.80 (0.2–2.1)	3.8	4.6	8.8
Water (mg./ml.)	987 (970–990)	987 (968–995)	984 (980–1000)	987 (960–1005)	962	958	949
pH	—	7.54 (7.42–7.70)	7.57 (7.40–7.80)	7.58 (7.50–7.70)	7.44	7.56	7.50
Total Nitrogen (mg./ml.)	0.41 (0.30–0.56)	0.78 (0.64–0.90)	0.41 (0.36–0.51)	0.33 (0.2–0.51)	2.53	2.84	3.06
Protein (mg./ml.)	—	3.49 (3.22–3.68)	2.36 (2.20–2.64)	2.46 (2.20–2.80)	15.54	18.70	25.0
Inorganic Phosphorus (mg./ml.)	0.02 (0.01–0.03)	0.07 (0.03–0.11)	0.02 (0.01–0.04)	0.02 (0.01–0.04)	0.02	0.03	0.02
Sodium (mEq/l.)	123 (100–158)	113 (107–119)	108 (93–120)	114 (105–140)	123	118	119
Potassium (mEq/l.)	42 (33–55)	37 (36–38)	49 (49–51)	56 (53–61)	41	46	43

* Each value represents the mean of at least 3 determinations (range).
† Single determinations.

and phosphorus (Table 1). Though estradiol stimulation resulted in similar water and pH values, the amounts of total nitrogen, protein and phosphorus were greater than observed in fluids from estrone or estriol treated animals. The values for water content and pH are comparable to those reported for fluid obtained from stilbestrol-stimulated rats (11). Average pH values for uterine fluids from other species include dog, 6.09 (11) and rabbit, 7.78 (11). Hall (27) reported an increase in pH of uterine fluid from rats and mice upon the onset of estrus. Low levels of phosphorus have also been noted in dog and rabbit uterine secretions (11).

Fluid from normal animals whose uteri had been ligated differs from that obtained from rats in proestrus or receiving estrogens as evidenced by the lower water content and higher concentration of nitrogen and protein (Table 1). No differences were noted for either pH or phosphorus content. Though discussion of data involving a single determination must be recognized as tenuous, it is apparent that extension of the period of ligation results in increased nitrogen and protein concentrations.

The distribution of electrolytes in all samples of luminal fluid studied indicates that it is more than a mere ultrafiltrate of plasma. Mean values of analyses are presented in Table 1. The mean concentration of sodium in uterine fluid for all treatments ranged from 108 to 123 mEq/l. (plasma 110–140 mEq/l.). Potassium concentrations, on the other hand, were approximately ten times those found in plasma (4 mEq/l.). Uterine fluid concentrations of sodium and potassium are comparable to those previously reported by Howard and De Feo (20).

A comparative study was made of the biologically active proteins, β-glucuronidase and alkaline and acid phosphatases in uterine fluids o

TABLE 2. ENZYMATIC ACTIVITY OF RAT UTERINE LUMINAL FLUID*

Enzyme	Treatment			
	Estrone	Estriol	Ligated (45 days)	Plasma
β-Glucuronidase†	15 (13–18)	6 (4–7)	89 (27–230)	1.3 (0.6–3.0)
Alkaline Phosphatase‡	651 (465–1218)	261 (196–482)	1016 (865–1190)	148 (84–186)
Acid Phosphatase‡	none	none	none	—

* Each value represents the mean of six determinations (range).
† Micrograms phenolphthalein liberated by one ml. of luminal fluid in one hour at 38° C from phenolphthalein glucuronide at pH 4.5.
‡ Micrograms of phosphorus liberated by one ml. of luminal fluid.

estrone and estriol-treated rats and also from rats with ligated uteri (Table 2). Acid phosphatase was absent from all samples of uterine fluid analyzed. Plasma acid phosphatase was not measured. Plasma values for β-glucuronidase and alkaline phosphatase were lower than those observed for any of the above mentioned treatments. The β-glucuronidase and alkaline phosphatase activities were higher in the fluids of estrone than in estriol-treated animals. Fluid from ligated uteri exhibited further elevated enzymatic activities.

The mobilities of the various uterine fluid and plasma protein fractions, along with their relative concentrations, are shown in Table 3. All values were calculated from the descending electrophoretic patterns. Electrophoretic patterns for plasma (Fig. 1A and B) show migrations for 60 and 120 minutes, respectively. Mobilities for plasma protein fractions (Table 3) are in substantial agreement with those previously reported (28, 29). In a more recent publication (30), however, mobilities for alpha-1 globulin and albumin were significantly greater than those previously reported.

The patterns for luminal fluids (Fig. 1C, D, E, F) show four to five peaks which, for purposes of comparison, have been numbered one to five in order of increasing mobility (Table 3). Fluids from estrone-treated rats and ligated uteri fractionated into five components, while that from animals given estriol show four peaks. However, regardless of the treatment, the respective mobilities of the protein components in luminal fluid are in good agreement (Table 3). The mobilities of components 1, 2, 3 and 4 for all treatments were pooled and found not to differ significantly ($P>0.05$) from beta, alpha-2, alpha-1 globulins and albumin of rat blood plasma, respectively. Component 5 of uterine fluid migrates with a mobility significantly exceeding that of albumin.

A comparison of the percentage composition of the protein components of uterine fluid indicates considerable variation, not only between treatments but also between samples on a single treatment (Table 3); therefore, conclusions must, at best, merely be considered subjective. The percentage composition of fluid from estriol and estrone-treated rats and animals with

TABLE 3. ELECTROPHORETIC FRACTIONATION OF RAT UTERINE LUMINAL FLUID

Treatment	Mobilities* Component number					Composition (%) Component number				
	1	2	3	4	5	1	2	3	4	5
Estrone	2.7 2.4	3.9 3.0	4.6 4.7	5.8 6.4	7.4 7.1	9 7	16 17	9 24	24 38	34 14
Estriol	— —	3.9 4.1	4.9 5.0	6.3 6.1	6.8 7.3	— —	15 21	25 9	23 32	37 38
Ligated (90 days)	2.7 3.2	3.8 4.2	5.2 5.4	6.4 6.1	7.9 7.7	5 8	16 19	15 16	17 11	40 37
Ligated (150 days)	— 2.2	3.9 2.7	5.4 4.7	6.2 5.7	7.6 7.3	— 18	6 9	11 11	50 44	15 18
Ligated (225 days)	2.7 2.4	3.2 3.0	4.9 4.9	5.7 5.7	7.1 6.9	24 19	8 5	13 9	44 48	11 19
Pooled Data	2.6 ± 0.3**	3.6 ± 0.5	5.0 ± 0.3	6.0 ± 0.3	7.3 ± 0.4					
	γ Globulin	β Globulin	$\alpha 2$ Globulin	$\alpha 1$ Globulin	Albumin	γ Globulin	β Globulin	$\alpha 2$ Globulin	$\alpha 1$ Globulin	Albumin
Rat Plasma	1.7 ± 0.1	2.8 ± 0.2	4.1 ± 0.2	5.2 ± 0.1	5.8 ± 0.1	4.5 ± 1.0	14.3 ± 1.6	6.9 ± 1.5	17.9 ± 2.1	56.4 ± 2.2

* Mobilities derived from the descending patterns are expressed as sq. cm. per second per volt $\times 10^5$.
** Mean ± Standard error of the mean.

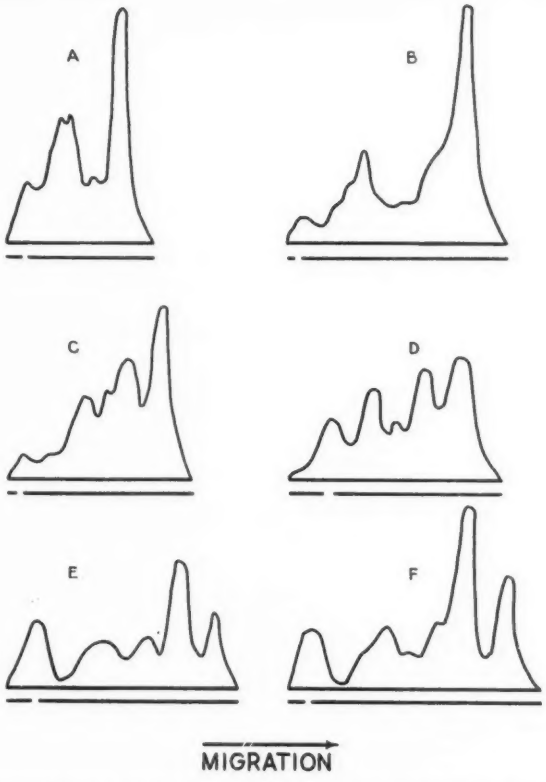

FIG. 1. Electrophoretic patterns of rat plasma and uterine luminal fluids: (A) rat plasma, 60 min.; (B) rat plasma, 120 min.; (C) luminal fluid, estrone treated animals, 60 min.; (D) luminal fluid, estriol treated animals, 60 min.; (E) luminal fluid, 5 month double ligated uteri, 90 min.; (F) luminal fluid, $7\frac{1}{2}$ month double ligated uteri, 120 min. The space in the solid lines beneath the patterns indicates the center of the salt anomaly.

uteri ligated 90 days appear comparable; however, as the period of ligation is extended, the percentages of peaks 1 to 4 approach those of beta, alpha-2, alpha-1 globulins and albumin of plasma. There also is a decrease in the percentage composition of component 5.

If one sums the percentage composition of peaks 4 and 5 for each treatment, the resultant mean of the sums is 59% (range 48–70); approximately the percentage composition of albumin in plasma. Though this similarity may be fortuitous, it may also suggest that albumin is in some way altered during its passage through uterine tissue into the lumen. Enzymes capable of attacking proteins have been demonstrated in rabbit and human uteri by Smith (31). These are of the type which have been designated exopeptidases; i.e., enzymes which are capable of hydrolyzing peptide bonds adjacent to free terminal amino acid carboxyl groups.

DISCUSSION

Though the literature on the physiology of reproduction contains numerous reports of the accumulation of fluid in the uteri of various mammalian species, definitive data on the origin of such fluids are absent. These experiments were conducted with the twofold purpose of determining 1) the chemical composition of rat uterine luminal fluid and comparing these constituents with those measured in blood plasma, and 2) the influence of estrone, estradiol, estriol and uterine ligation on the formation and composition of uterine fluid.

Estradiol (1 μg./day/3 days) resulted in a mean volume of 0.24 ml. and estrone (5 μg./day/3 days) in a mean volume of 0.38 ml. (Table 1). Estriol (0.5 mg./day/3 days), however, produced a mean volume of fluid of 0.80 ml. Though the total dose of estriol is 100 to 500 times the total of estrone or estradiol administered, respectively, increasing the dosage of the latter two estrogens did not potentiate the accumulation of fluid. Though dosage is an important factor in these experiments, attention must also be directed to the nature of action exhibited by these three estrogens. Hisaw (32) has pointed out that, in general, the actions of estrogens on the uterus are the same; however, each has its individual peculiarities. For instance, equilenin and estriol are strong promoters of fluid imbibition but are relatively weak stimulators of uterine growth. Moreover, Szego and Roberts (33) found that in immature rats, the relative effectiveness of estrogens in promoting the uptake of water by the uterus was of the order of estriol, estradiol and estrone.

Considerable amounts of uterine luminal fluid can be obtained from animals ligated either at the cervix or at both the cervix and uterotubal junction. Data in this report tend to indicate that the accumulation of fluid is proportional to the period of ligation. Though most studies have been conducted with animals with intact ovaries, several reports have indicated that ligated uteri in castrated animals become distended with fluid (12, 13, 14).

Bishop (12) has suggested that no fluid is normally contributed to the rabbit genital tract from either the peritoneal cavity or the uterus, but that the site of origin is the oviduct. This conclusion is based upon the inability of doubly ligated uterine horns of estrus or pregnant does to accumulate significant quantities of fluid. Conversely, Markee, *et al.* (5), in experiments in which the right uterine horn of rabbits was ligated close to each of its ends, demonstrated distention by accumulation of fluid. Uterine fluid accumulation has also been demonstrated in dogs prepared by proximal and distal ligation of the uterine horns (11).

In the studies reported herein, it is possible that uterine fluid obtained from castrated rats treated with estrogens, and in which the uteri were not ligated, could have originated in both the oviducts and uteri. However,

the possibility of fluid originating from the oviducts was obviated in those experiments in which ligatures were placed at both the cervix and uterotubal junction.

The low levels of protein in luminal fluid (0.25–0.35%) collected from estrogen-treated castrated rats and also rats during proestrus resemble those of cerebrospinal fluid (34) and prostatic secretion (35). Uterine fluids from the dog and rabbit were also reported to have a low protein content (11); however, these fluids were collected from animals having ligated uteri. Fluid from rats having ligated uteri was appreciably higher in protein than that observed in animals receiving estrogens; moreover, as the period of ligation was extended, the concentration of protein increased (Table 1).

Electrophoretic analyses of uterine fluid proteins show a pattern similar to that of plasma, and also the presence of a protein component possessing a mobility exceeding that of albumin (Table 3). Though mobility values for protein components were reproducible in fluid samples regardless of treatment, percentage composition was extremely variable. Uterine fluid values for gamma globulin are not included in our tabulations because of considerable difficulty in separating it satisfactorily from the salt boundary.

A component in rat blood serum having a mobility greater than that of albumin (approximately 7.7) has been observed by Heim (30) and also by Shmerling and Uspenskia (36). The percentage composition of this component was less than 1%. This protein fraction was not observed in the electrophoretic patterns of plasma in studies reported herein.

The relative percentage of the rapidly moving component of uterine fluid is greater than that of albumin both in the fluid from estrogen-treated animals and from rats with uteri that had been ligated for 90 days. However, as the period of ligation is extended to 5 months, the fluid shows a decrease in the rapid component and the composition of the protein fractions more closely resembles that of plasma (Table 3). This change in composition may reflect the alterations in the uterine epithelial structures. With prolonged ligation, areas of the stroma of the endometrium are exposed, making it possible for interstitial fluid to enter directly into the lumen of the uterus.

It is evident that the activities of both alkaline phosphatase and β-glucuronidase in uterine fluids are substantially higher than those of plasma, lending further support to the hypothesis that rat uterine luminal fluid is more than a transudate of blood. The estrogens also appear to differ in their ability to induce elevated enzyme levels in uterine fluid (Table 2). While estriol is more efficacious in the promotion of fluid imbibition than estrone, it is less capable of increasing enzyme activity. These differences in enzymatic activity may again reflect the relative effectiveness of these steroids to stimulate uterine growth. It has been suggested

that the increase in uterine activity of β-glucuronidase during estrogen stimulation is associated with growth of the uterus (37). Moreover, the injection of estrogens has been shown to induce an increase in the alkaline phosphatase of uterine epithelium, circular muscle and glands (38).

The absence of acid phosphatase in uterine fluid was not surprising. Deane and Dempsey (39) histochemically localized acid phosphatase in the Golgi region of the uterine epithelium of the pregnant cat and cow; however, this phosphatase has not been observed in the uterine epithelium of the guinea pig, rat, mouse, or human (40).

Sodium and potassium concentrations in rat uterine luminal fluid collected during proestrus was the subject of a report by Howard and De Feo (20). The potassium ranged from 35 to 40 mEq/l. Howard and De Feo (20) conclude that "the fluid in the resting uterine lumen is a secretion rather than a transudate from plasma" since sodium in plasma and uterine fluid is similar while potassium levels are elevated some tenfold. One point requiring further investigation to prove this hypothesis is an explanation as to why the fluid from animals ligated 4 or 5 months retains its high potassium concentration, since by histological assessment, the glandular epithelium has disintegrated. It must also be remembered that this high concentration of potassium may again be a reflection of changes occurring in the surrounding uterine tissue, since Cole (41) has reported a pronounced increase in intracellular potassium in the uteri of rats following the injection of estradiol.

Acknowledgment

The author wishes to thank Dr. F. L. Hisaw for his interest and support of this work. Gratitude is expressed to Dr. A. G. Olson, Brandeis University, Waltham, Massachusetts for suggesting studies of fluids from ligated uteri and also for the histological evaluation of such uteri.

REFERENCES

1. Long, J. A. and H. M. Evans: *Mem. Univ. Calif.* **6:** 1. 1922.
2. Allen, E.: *Am. J. Anat.* **42:** 467. 1928.
3. Parkes, A. S.: *The Internal Secretions of the Ovary;* Longmans, Green and Co., New York. 1929.
4. Brambell, R. W. and I. R. Rowlands: *Phil. Trans. Roy. Soc.* **B226:** 77. 1935.
5. Markee, J. E., W. M. Wells and J. C. Hinsey: *Anat. Rec.* **64:** 221. 1935–36.
6. Hartman, C. G.: *Am. J. Anat.* **32:** 353. 1923.
7. De Marco, R.: *Arch. di fisiol.* **29:** 181. 1930.
8. Howard, E.: *J. Cell and Comp. Physiol.* **3:** 291. 1933.
9. Sturgis, S. H.: *Endocrinology* **31:** 664. 1942.
10. Olds, D. and N. L. Van Demark: *Am. J. Vet. Res.* **18:** 587. 1957.
11. Shih, H. E., J. Kennedy and C. Huggins: *Am. J. Physiol.* **130:** 287. 1940.
12. Bishop, D. W.: *Am. J. Physiol.* **187:** 347. 1956.
13. Reynolds, S. R. M. and S. Kaminester: *Anat. Rec.* **69:** 281. 1937.
14. Homburger, F., M. S. Grossman and A. Tregier: *Proc. Soc. Exper. Biol. and Med.* **90:** 719. 1955.

15. Warren, M. R.: *Am. J. Physiol.* **122:** 602. 1938.
16. Homburger, F., A. Tregier and M. S. Grossman: *Endocrinology* **61:** 634. 1957.
17. Ringler, I.: *Proc. Soc. Exper. Biol. and Med.* **95:** 506. 1957.
18. Astwood, E. B.: *Endocrinology* **23:** 25. 1938.
19. Broome, A. W. J. and G. E. Lamming: *J. Endocrinology* **18:** 220. 1959.
20. Howard, E. and V. J. De Feo: *Am. J. Physiol.* **196:** 65. 1959.
21. Ringler, I.: *Fed. Proc.* **15:** 496. 1956.
22. Lewis, L. A.: *Electrophoresis in Physiology;* Thomas, Springfield, Ill. 1950.
23. Gornall, A. G., C. J. Bardawill and M. M. David: *J. Biol. Chem.* **177:** 751. 1949.
24. Sumner, J. B.: *Science* **100:** 413. 1944.
25. Talalay, P., W. H. Fishman and C. Huggins: *J. Biol. Chem.* **166:** 757. 1946.
26. Schmidt, G. and S. J. Thannhauser: *J. Biol. Chem.* **149:** 369. 1943.
27. Hall, B. V.: *Physiol. Zool.* **9:** 471. 1936.
28. Moore, D. H.: *J. Biol. Chem.* **161:** 1945.
29. Cohen, P. P. and F. L. Thompson: *J. Lab. and Clin. Med.* **33:** 75. 1948.
30. Heim, W. G.: *Am. J. Physiol.* **197:** 135. 1959.
31. Smith, E. L.: *J. Biol. Chem.* **173:** 553. 1948.
32. Hisaw, F. J., Jr.: *Endocrinology* **64:** 276. 1959.
33. Szego, C. M. and S. Roberts: *Rec. Prog. Hormone Research* **8:** 419. 1953.
34. Flexner, L. B.: *Physiol. Rev.* **14:** 161. 1934.
35. Mann, T.: *The Biochemistry of Semen.* John Wiley and Sons, New York. 1954.
36. Shmerling, Z. G. and V. D. Uspenskia: *Biokhimiya* **20:** 31. 1955.
37. Levvy, G. A.: *Brit. Med. Bull.* **9:** 126. 1953.
38. Jeener, R.: *Biochim. et biophys. acta.* **2:** 439. 1948.
39. Deane, H. W. and E. W. Dempsey: *Anat. Rec.* **93:** 401. 1945.
40. Wislocki, G. B. and E. W. Dempsey: *Am. J. Anat.* **77:** 325. 1945.
41. Cole, D. F.: *J. Endocrinology* **7:** 12. 1950–51.

THE "LIGHT CELLS" OF THE THYROID GLAND IN THE RAT[1]

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ABSTRACT

Shortly after an injection of thyrotrophic hormone, all follicular cells of the thyroid gland of the rat contain numerous intracytoplasmic colloid droplets, whereas a few cells—referred to as light cells—do not contain any. In the young rat, this brief thyrotrophic hormone treatment is essential for the identification and counting of light cells. In the old rat, however, the presence of many colloid droplets and densely stained material in its follicular cells makes the use of the hormone superfluous.

With most staining techniques, the light cells show a large, pale nucleus and a faintly stained cytoplasm. They are all located within follicles, but are not in contact with the colloid. They lie singly or in small groups between the follicular cells and the basement membrane, or among the follicular cells, but even in this case they are separated from the colloid by a layer of follicular cell cytoplasm.

The light cells are distributed in the two thyroid lobes, chiefly in the follicles of the central region, but are absent from the isthmus. Their number is small, one or a few per cent of the total population of epithelial cells. This proportion does not change with age, so that the populations of follicular and light cells must increase at the same rate throughout life.

Since light cells are within follicles, they are considered to be epithelial in nature and, therefore, are neither ganglionic cells, parathyroid cells nor embryonic cell rests, as assumed by some authors. The light cell may be looked upon as a cell species in its own right, although it is possible that it originates from the follicular cell.

SINCE the thyroid is composed of follicles embedded in connective tissue, the cell population of the gland is composed of epithelial cells lining the follicle—or *follicular cells*—and of connective tissue cells. In addition it has been repeatedly reported (see review in ref. 21) that large, pale staining cells—hereafter referred to as *light cells*—may also be present. However, the existence of these cells has been questioned (21). Indeed, out of five histology textbooks published after 1950 (4, 10, 13, 14, 23), only two mention light cells (13, 14).

Our group became interested in the problem when thyroid tumors were

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induced in rats fed a low iodine diet for about a year (2, 17, 18). The tumors were mainly of two types: one composed of polarized cells bearing a resemblance to, and therefore assumed to originate from, the follicular cells, and the other composed of large, pale, unpolarized cells, the origin of which was not known. However, the thyroids of the control rats ingesting an adequate amount of iodine showed not only the follicular cells, which in these old animals were heavily stained and rich in colloid droplets, but also large cells with pale nuclei and a lightly stained, droplet-free cytoplasm, which appeared to be the cells described by previous authors under various names (21) and referred to here as light cells. They were similar to the cells making up the second type of tumor and therefore were felt to be the source of these tumors (2, 17, 18).

While the contrast with heavily-stained and droplet-laden follicular cells made light cells conspicuous in old rats, young animals had few droplets and little heavily stained material in their follicular cells, so that all cells presented more or less the same appearance. Very few of the cells could be considered to be light cells, and therefore it was not obvious whether or not light cells occurred in all young rats.

Some time ago, de Robertis showed that colloid droplets are readily induced in thyroid cells by a single, large dose of thyrotrophic hormone (7). It was reasoned that, under these conditions, light cells might not produce colloid droplets, and thus would be easily distinguished from the droplet-containing follicular cells. This was found to be the case, and the absence of droplets became the basis of a method for light cell identification and investigation.

MATERIAL AND METHOD

Identification of light cells

Thyrotrophic hormone (Nordic Biochemicals, Montreal) was dissolved in distilled water and injected intraperitoneally into female albino rats (Holtzman). The animals were sacrificed by exposure to ether, and the trachea, with the thyroid attached, was fixed in Bouin's fluid, sectioned and stained with periodic acid-Schiff and hematoxylin. The thyroids were examined in animals sacrificed after administration of various doses of hormone and at various times after injection, as indicated in the results.

Quantitative estimation of light cells

From each thyroid, a group of 3 serial sections, 5 or 6 μ in thickness, was stained with periodic acid-Schiff and hematoxylin. Photomicrographs of the center of the second section were made by direct projection of the slide on photographic paper (bromide printing paper) so as to obtain a negative image ($\times$400) showing every nucleus individually.

Each cell appearing on the second section (except of course connective tissue cells) was examined under the microscope. If the cytoplasm contained colloid droplets, its nucleus was recorded on the photograph as belonging to a follicular cell. If it did not, the cell under study was traced in the two adjacent sections, and if again no colloid droplets were seen, it was recorded as a light cell.

In each thyroid a total of 1500 to 4000 nuclei were thus identified and recorded on photographs. The ratio of light to follicular cells was then calculated.

RESULTS

Thyroid glands of young and old untreated rats

Examination of thyroid glands of *young* untreated female rats, aged one month (121–132 gm. body weight) or 4 months (220–260 gm. body weight), revealed the well known arrangement in follicles; each one of them was separated from connective tissue by a basement membrane. As in other locations, the basement membrane is a thin layer of connective tissue material separating the epithelial cells of the follicle from the surrounding loose connective tissue and blood vessels. It differs from this connective tissue by its stainability with periodic acid-Schiff and reticular fiber techniques (11). Within the basement membrane, cuboidal or columnar epithelial cells surrounded the "colloid" filling the lumen (Fig. 1). The nuclei of these cells were moderately basophilic, while the slightly basophilic cytoplasm had a clear appearance and occasionally contained a few minute spherical inclusions staining purple with the periodic-acid-Schiff technique—the colloid droplets (arrow D, Fig. 1). Under proper staining conditions, the apical region was characterized by the minute cross-sections of the terminal bars at the junction with neighboring cells. The basal region, which contained the nucleus, was in contact with the basement membrane. These cells were, therefore, polarized. They were the *follicular cells*. Occasionally, these cells appeared in multiple layers, as would be expected in tangentially sectioned follicles.

In a few follicles, small groups of cells lying between a layer of follicular cells and the basement membrane showed a large nucleus and a rounded outline, but otherwise did not differ much from the other cells. They were not conspicuous since their cytoplasm had a pale appearance similar to that of the other cells, and for the majority of them, it could not be decided on histological examination whether they were similar to or different from follicular cells.

In the thyroid glands of *old* rats, aged 14 months (305–355 gm. body weight), the cells in contact with the colloid contained numerous colloid droplets (Fig. 3) and had a more heavily stained cytoplasm (Fig. 4) than in young rats, but like those in young rats they were cuboidal or columnar and displayed terminal bars whenever two of them joined. These cells were obviously follicular cells.

The thyroids of old rats also had cells of another type, which did not contain colloid droplets and were larger on the average than follicular cells (Fig. 3, L). Instead of a well defined cuboidal or columnar arrangement, they were not polarized, displayed no terminal bars and their shape varied considerably. They often had a smooth circular or oval outline (Fig. 4) but occasionally were polygonal or spindle shaped (Fig. 5). When these cells lay singly among follicular cells, their limits were precise (Fig. 5), but when they were arranged in groups, the cell membranes separating them from

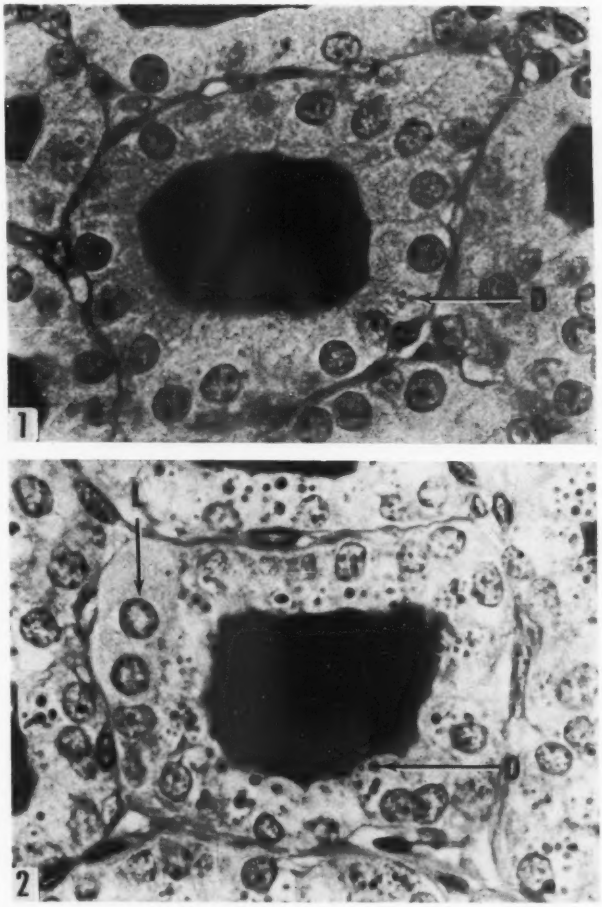
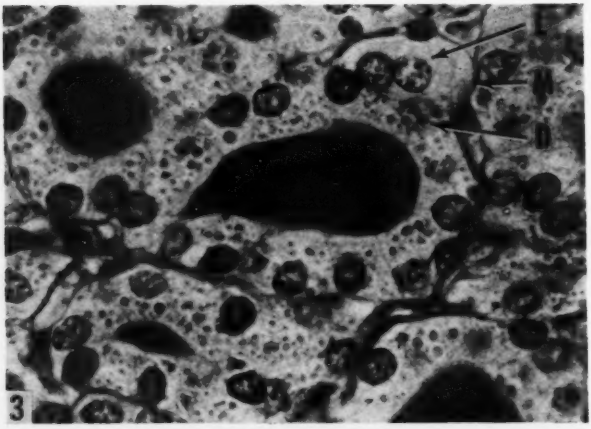


FIG. 1. Thyroid of an untreated 3 month old rat. The center of the picture shows a follicle containing dark staining colloid and surrounded by a basement membrane. The intracytoplasmic colloid droplets (D) are few. Periodic acid-Schiff and hematoxylin. ×861

FIG. 2. Thyroid of a 3 month old rat 90 minutes after thyrotrophic hormone injection. The follicular cells contain abundant colloid droplets (D). On the left, two light cells (L) are contrasting by their lack of droplets. Periodic acid-Schiff and hematoxylin. ×861

one another were hazy (Fig. 4). Their nuclei were round and usually larger than those of epithelial cells (Fig. 4) and often contained an unstained vacuole. Mitoses of these cells were seen, but only seldom. Their cytoplasm was lightly stained, did not contain colloid droplets and appeared homogenous, although a small basophilic mass was occasionally seen. These unpolarized cells were the *light cells*.

The light cells were located among the droplet-laden follicular cells or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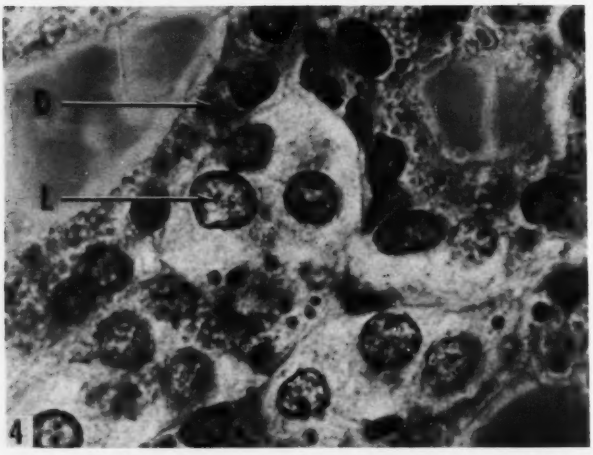
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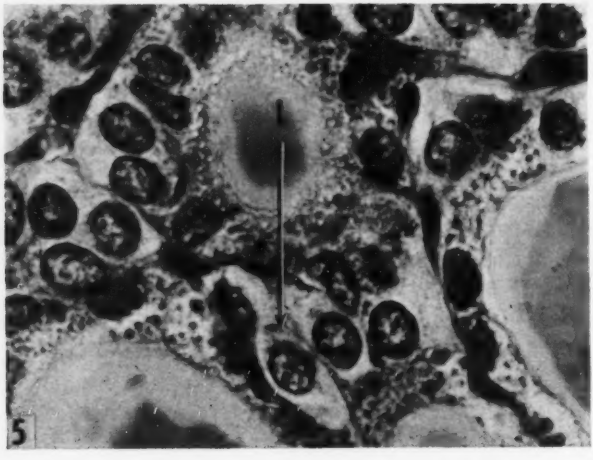
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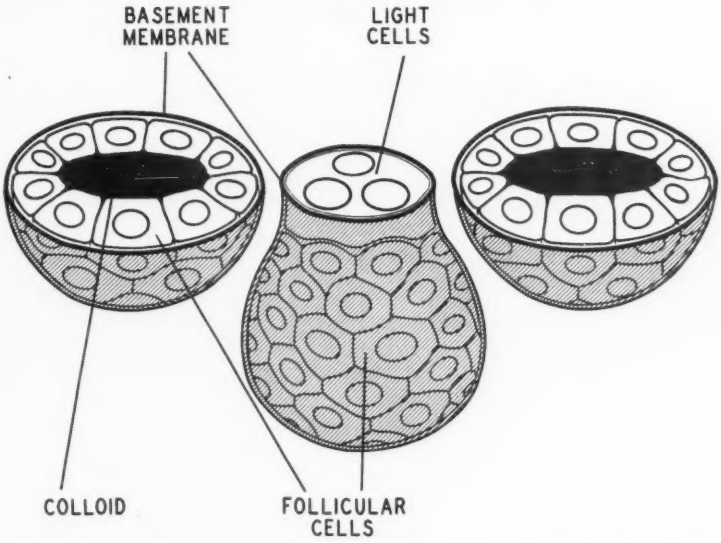

FIG. 6. Schematic representation of a group of light cells which, in the plane of section appear as a discrete cluster of 3 cells surrounded by a basement membrane and located between follicles (in apparent inter- or parafollicular position). However the basement membrane enveloping these cells is continuous with that of the follicle situated immediately below. Thus the 3 light cells are not inter- or parafollicular, but are really intrafollicular. This was found to be true in all instances.

between these cells and the basement membrane. They were separated from direct contact with the colloid by a whole follicular cell or by a layer of its cytoplasm. Occasionally, a section showed a group of light cells appearing to be in an interfollicular position. A basement membrane surrounded such a group. When three dimensional models were built from serial sections (19), the basement membrane was found to be continuous with that of a follicle, so that these apparently discrete groups of light cells were actually contained within a follicle (Fig. 6).

Thyroid glands of young rats injected with thyrotrophic hormone

One month old rats (120–135 gm. body weight) and 4-month old rats (200–270 gm. body weight) were injected respectively with 3 and 5 U.S.P.

FIGS. 3–5. Thyroid of an untreated 15 month old rat. The follicular cells contain abundant colloid droplets (D). In Fig. 3, upper right, the light cells (L) are inside the basement membrane (M) of the follicle.—Fig. 4 shows large round light cells (L) in the center, while Fig. 5 shows spindle-shaped light cells (L). The heavy staining of follicular cells in old rat is shown in Fig. 4 in the region at the arrow D and in the follicle at upper right.

Fig. 3: periodic acid-Schiff and hematoxylin. ×861

Figs. 4 and 5: Masson trichrome. ×975

units thyrotrophic hormone and sacrificed one and a half hours later. The follicular cells retained their shape, but were slightly enlarged and bulging at the apex. Their cytoplasm exhibited numerous colloid droplets which stood out clearly. Other cells were completely free of colloid droplets and displayed the characteristic features of light cells as seen in old animals (Fig. 2, L). It was concluded that, while light cells were not readily seen in untreated 1- and 4-month old rats, their presence could be demonstrated by the lack of droplets after thyrotrophic hormone injection. In fact, the identification of light cells after thyrotrophic hormone treatment was found to be so reliable that they could be counted accurately. The light cell problem could therefore be investigated objectively by quantitative methods.

Colloid droplet response and light cell identification at various time intervals after thyrotrophic hormone injection

Since the method for identifying light cells relied on their lack of colloid droplets, it was important that the thyroid be examined under conditions when the follicular cells would contain a large number of droplets. The optimum time at which this occurs after thyrotrophic hormone injection was determined as follows.

Five groups of three-month old (208–242 gm. body weight) albino rats were each given a single intraperitoneal injection of thyrotrophic hormone (20 U.S.P. milliunits per gm. body weight) and sacrificed 30, 60, 90, 180 and 360 minutes later respectively. A sixth group serving as control was given distilled water and sacrificed 30 minutes later. Transverse section from the middle portion of the thyroid were examined in the usual manner. Both light and follicular cells were counted on sections from animals sacrificed at 90 and 180 minutes. The number of colloid droplets was determined by a modification of the method advocated by Del Conte and Vasena (5). Three follicles among the richest in droplets were selected from each lobe. The colloid droplets in each follicle were then counted with the microscope focused on the plane with the greatest number of colloid droplets. The number of nuclei in the follicle was recorded and the average number of droplets per nucleus was calculated.

The results (Table 1) showed that the concentration of colloid droplets increased steadily up to 3 hours after thyrotrophic hormone injection and then decreased. Thus, the colloid droplet response was most intense and uniform during the period extending from $1\frac{1}{2}$ to 3 hours after thyrotrophic hormone injection. During this period all cells which were free of droplets also showed the other characters of light cells, and these cells could be readily identified. Estimation of the frequency of these cells at $1\frac{1}{2}$ and 3 hours yielded very close figures, namely 2.65 and 2.57 light cells respectively for 100 follicular cells. Since towards the third hour and later the shape of the cells and of the follicles was occasionally distorted, the interval of $1\frac{1}{2}$ hours was selected for routine work.

TABLE 1. COLLOID DROPLET RESPONSE AT VARIOUS TIMES AFTER INJECTION OF 20 U.S.P. MILLUNITS PER GM. BODY WEIGHT OF THYROTROPHIC HORMONE

Time after thyrotrophic hormone injection (minutes)	Number of rats	Number of droplets per cell	
		Mean ± S.E.	P*
control	8	0.44 ± 0.24	
30	8	2.58 ± 0.25	<0.001
60	7	2.78 ± 0.54	0.001–0.01
90	8	4.53 ± 0.58	<0.001
180	6	6.25 ± 0.30	<0.001
360	6	3.49 ± 0.27	<0.001

* Comparison with controls.

Colloid droplet response and light cell identification after various doses of thyrotrophic hormone

The minimal dose of thyrotrophic hormone producing enough colloid droplets in follicular cells for a reliable enumeration of light cells was determined in 3-month old rats. A preliminary experiment showed that increasing the dose from 20 to 80 mu. per gm. body weight did not increase the intensity of the response nor improve the conditions for counting light cells. Then lower doses were investigated. Six groups composed of 6 rats each weighting from 192 to 238 gm. were given a single injection of 0, 0.001, 0.1, 1.0 and 10 mu. thyrotrophic hormone per gm. body weight respectively, and were sacrificed $1\frac{1}{2}$ hours thereafter. Sections from the middle portion of the thyroid were prepared and stained in the usual manner.

In the thyroids of the rats receiving 0.01 mu. thyrotrophic hormone per gm. or less, very few droplets were seen (0.8–0.9 per cell in the richest follicles; Table 2). The majority of follicular cells did not contain any and only a few light cells could be identified with certainty.

In the animals receiving 0.1 mu. per gm. body weight, the droplets were more abundant (2.4 droplets per cell in the richest follicles) and were more uniform than with 0.01 mu. But not all follicular cells showed droplets, so that light cell identification was not fully reliable. With 1.0 mu. thyrotrophic hormone however, the response was very intense and uniform (6.4 droplets per cell) and the light cells could be readily recognized. A higher dose of thyrotrophic hormone (10 mu.) did not significantly increase the

TABLE 2. COLLOID DROPLET RESPONSE AFTER VARIOUS DOSES OF THYROTROPHIC HORMONE

Dose thyrotrophic hormone (U.S.P. milliunits per gm. body weight)	Number of droplets per cell	
	Mean ± S.E.	P*
0	0.83 ± 0.27	
0.001	0.91 ± 0.23	0.8 –0.9
0.01	0.88 ± 0.24	0.8 –0.9
0.1	2.42 ± 0.31	0.001–0.01
1.0	6.37 ± 0.94	0.001
10.0	7.00 ± 0.58	0.001

* Comparison with controls.

droplet response, nor improve the conditions for the counting of light cells.

These results indicate that the minimum dose inducing a colloid droplet response intense enough for a reliable estimation of the light cell number lies between 0.1 and 1.0 mu. thyrotrophic hormone per gm. body weight. In fact, in another experiment a dose of 0.6 mu. produced a droplet response as intense as at the 1.0 level, and yielded a figure for light cells which was not significantly different from that obtained by injection of 20 mu. per gram (Table 5). Therefore the dose of 1.0 mu, per gm. body weight has been used in recent experiments and was found adequate.

Distribution of light cells in the thyroid gland

It has been mentioned repeatedly in the literature (8, 27, 29) that light cells are not evenly distributed in the gland, but predominate in the center of the lobes. Since this conclusion was based on qualitative observations, it was decided to reexamine the distribution of light cells using our quantitative method. Six rats were injected with thyrotrophic hormone (20 mu. per gm. body weight) and sacrificed 90 minutes thereafter. Transverse serial sections of the whole gland were cut at 6 μ. At every fiftieth section (that is, every 300 μ), a set of three consecutive sections was mounted and stained. Light cells were then counted in three sets taken at 900 μ intervals along the longitudinal axis of a lobe in each animal, one set halfway between the two poles, one near the cephalic pole and the last one near the caudal pole (Fig. 7). The entire thyroid sections were reproduced as $3' \times 2'$ photographs, on which the position of all light cells was then recorded.

Comparison of the three sets showed the highest proportion of light cells halfway between the 2 poles (about 1 light cell per 100 follicular cells). Near the caudal pole there were only 0.34 light cells per 100 follicular cells and near the cephalic pole there were almost none (Fig. 7). No light cells were found in the isthmus joining the two lobes.

Within the section situated halfway between the poles, light cells were rather scarce in peripheral follicles and common in the central area (Fig. 8) where they were chiefly seen amongst small and medium sized follicles although some were in large ones. The few light cells found at the periphery were usually not in the follicles located immediately beneath the capsule, but in deeper ones. In summary, light cells were found essentially, although not exclusively, in the follicles making up the core of the thyroid gland.

From the figures obtained at the 3 levels, it was calculated that the average proportion of light cells to follicular cells throughout the entire thyroid was in the order of 0.7%. Thus, light cells constitute a very small fraction of the total thyroid parenchyma. In the center of the thyroid of the normal rat the proportion has been found to be consistently between 1 and 2%. However, for some unknown reason, several experiments (Tables 3, 4, and 6) yielded consistent figures around 5%, a significantly higher level. Thus there may be some variation in the proportion of light cells to follicular cells in normal rats.

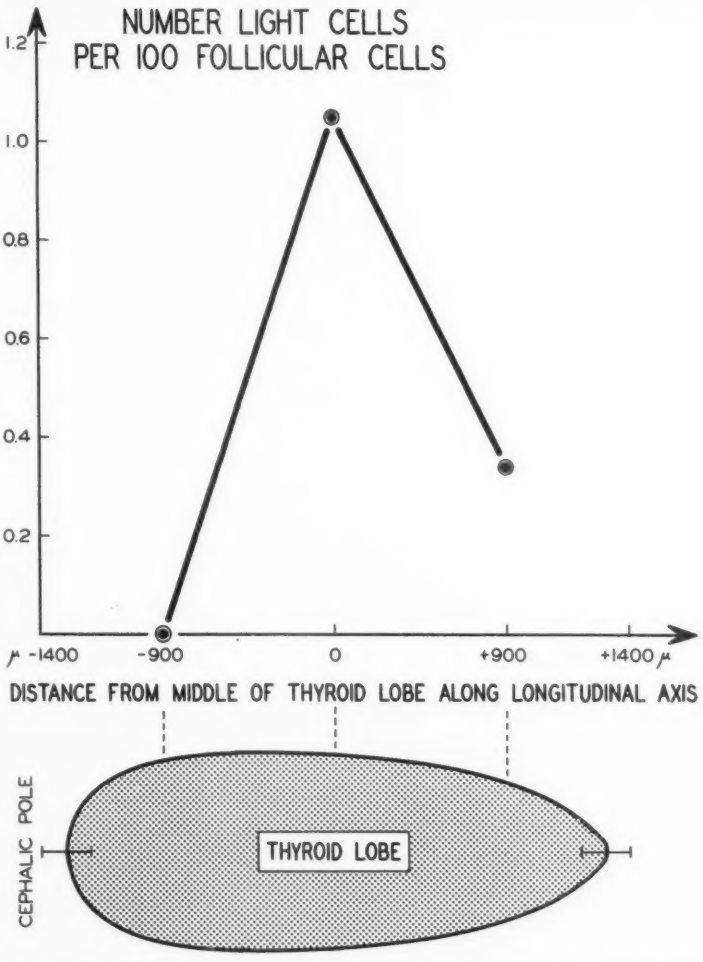

Fig. 7. Distribution of light cells in the thyroid gland along the longitudinal axis of the lobe. The highest concentration is found in the middle. Each figure plotted is the average for six animals.

Since the middle portion of the thyroid lobe is rich in light cells, while the poles are not, a careful standardization of the selection of sections to be compared is required for reproducible results to be obtained.

In relation with the light cell distribution we have examined the claim (12) that light cells are more abundant in the immediate vicinity of the ultimobranchial body. This problem was approached in two ways. The first one consisted in comparing the proportion of light cells to follicular cells in sections containing and not containing the ultimobranchial body. No correlation was found between the number of light cells and the presence of

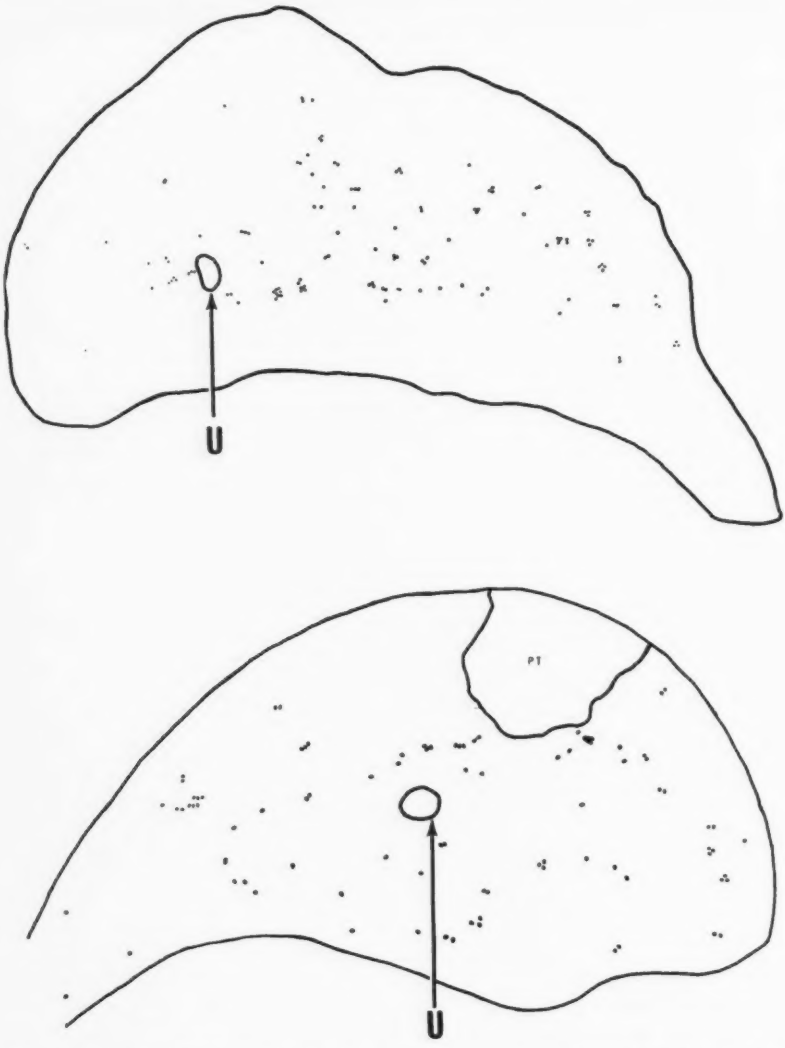

FIG. 8. Distribution of light cells in two entire sections of rat thyroid lobes. Each black dot (grossly enlarged) is a light cell. No light cells were found in the extreme periphery. Their distribution did not show a correlation with the position of the ultimobranchial body (U).

this structure (Table 3). The second approach consisted of mapping the light cells in entire transverse sections of the thyroid lobe showing the ultimobranchial body, and studying the distribution of light cells relative to the ultimobranchial body, as done in the diagrams in Fig. 8. Again no

TABLE 3. CONCENTRATION OF LIGHT CELLS IN THE VICINITY OF THE ULTIMOBRANCHIAL BODY

Thyroid tissue	Number of rats	Number of light cells per 100 follicular cells
		Mean ±S.E.
Control area	6	5.2 ±0.8
U.B.B. area	6	4.3 ±0.7

relationship was noted between the position of this structure and that of light cells.

Influence of thyrotrophic hormone on light cells

Since our method for estimating the number of light cells involves an acute treatment with thyrotrophic hormone, the question arises whether or not such hormonal treatment may change their number. In view of the short time (90 minutes) elapsing between hormone injection and sacrifice, such an effect appeared unlikely; but definite proof was necessary. Two approaches were used. The first one consisted in comparing old rats receiving and not receiving the hormone, since in old rats the number of colloid droplets is high and the light cells can be readily recognized and counted in the absence of thyrotrophic hormone treatment. Fifteen-month old rats weighing 350 gm. were subdivided into a group of untreated controls and another given 20 mu. thyrotrophic hormone per gm. body weight, and sacrificed 1½ hours later. The proportion of light cells to follicular cells was the same in both groups (Table 4).

The second approach was to inject young rats with two different doses of thyrotrophic hormone, on the assumption that the effect of this hormone on light cells, if any, should be more intense with a larger dose. 3-month old rats received either 0.6 mu. thyrotrophic hormone per gm. body weight, the lowest dose allowing reliable identification of light cells, or 20 mu. per gm. body weight. No significant difference was observed (Table 5).

These results indicate that in the experimental conditions used for identifying light cells, the proportion of these cells was not modified by the hormonal treatment. Thyrotrophic hormone merely induced colloid droplets in the follicular cells, so that the droplet-free light cells stood out by contrast.

TABLE 4. EFFECT OF THYROTROPHIC HORMONE OF THE PROPORTION OF LIGHT CELLS TO FOLLICULAR CELLS IN THE THYROID OF OLD RATS

Treatment	Number of rats	Number of light cells per 100 follicular cells
		Mean ±S.E.
None	6	5.9 ±2.3
Thyrotrophic hormone	7	5.5 ±0.8

TABLE 5. EFFECT OF THYROTROPHIC HORMONE ON THE PROPORTION OF LIGHT CELLS TO FOLLICULAR CELLS IN THE THYROID GLAND

Dose thyrotrophic hormone (U.S.P. milliunits per gm. body weight)	Number of rats	Number of light cells per 100 follicular cells	
		Mean ±S.E.	P
0.6	7	1.47 ±0.37	0.3–0.4
20.0	6	2.57 ±0.93	

Influence of age on light cells

The light cells are not so conspicuous in young untreated rats as in old animals; nevertheless they are present, as was found after the acute treatment with thyrotrophic hormone. However, it was possible that their inconspicuousness in young rats was not only due to the lack of colloid droplets in the follicular cells, but also to the fact that light cells were less abundant in young than in old rats. This question was investigated by examining the thyroid of rats of various ages, from birth to 15 months, given a dose of 20 mu. thyrotrophic hormone per gm. body weight 90 minutes prior to sacrifice.

In rats aged five weeks and more, thyrotrophic hormone elicited the usual abundant formation of colloid droplets and the light cells were readily counted. The proportion of light cells to follicular cells was the same whether the rats were aged 5 weeks, 5 months or 15 months (Table 6). In 3-week old rats, however, the droplet response to the hormone injection was less intense than in the older groups, but was still sufficient for light cell identification. In 8–10 day old rats injected with 20 mu. per gm. the droplet response was poor, whereas after a dose 10 times as high a fair number of droplets were present and the light cells could again be identified and counted. In these two younger groups the proportion of light cells was about the same as in older rats (Table 6). In new-born rats the droplet response was so poor even after injecting a dose of 200 mu. per gm. that light cells could not be counted.

In summary, these results indicate that the proportion of light cells to follicular cells did not vary between the ages of 8 days and 15 months.

TABLE 6. EFFECT OF AGE ON THE PROPORTION OF LIGHT CELLS TO FOLLICULAR CELLS IN THE THYROID GLAND

Age	Body-weight (gm.)	Number of rats	Number of light cells per 100 follicular cells (Mean ±S.E.)
8–10 days	10–15	6	4.5 ±1.0
3 weeks	30–50	5	5.8 ±0.5
5–7 weeks*	120–135	5	4.7 ±1.5
4–5 months*	230–270	5	5.2 ±0.7
13–15 months*	280–370	7	5.5 ±0.8

* Age as indicated by dealer.

DISCUSSION

In 1876 Baber (3) described in the thyroid of the dog "large rounded cells, each provided with an oval nucleus, found either singly or in groups amongst the epithelial cells." He gave them the name of "parenchyma" cells. Later these cells have been redescribed under various names, such as protoplasmareiche Zellen (16), parafollikuläre Zellen (1), embryonale Zellhaufen (36), Restzellen (15), parafollicular cells (24), grey cells (12), cellules souches (26), Makrothyreocyten (22), cellules claires (25), cellules cholinesterase positives (8), intercellular cells (21), light cells (2), and polyodocytes (25). The qualification parafollicular or interfollicular was given by various authors (1, 21, 24) to these cells since they often appear as a discrete group of cells located between the follicles. However, in three-dimensional models reconstructed from serial sections of the rat thyroid (19), it was shown that these apparently isolated groups of light cells lay inside the basement membrane of a follicle, as may be seen in Figure 6. Indeed not a single instance of light cells located outside a follicle has been found in the models.

A number of hypotheses have been proposed (21) concerning the nature of light cells. Some authors believe that they are mere tangential sections of follicular cells (21) or cells beginning mitosis (21). Others postulate that they originate from follicular cells (1, 24) or conversely that they may change into follicular cells to form new follicles (9, 36, 37) or that they are stem cells producing follicular cells (26). They have been considered to be embryonic rests or cells originating from embryonic rests, such as the ultimobranchial body or thyroglossal duct (8, 12); cells belonging to the "system of Feyrter" (25) which consists of cells of various endocrine organs showing histological similarities; secretory cells (31); parathyroid cells (32); Schwann cells or neurosecretory cells (30).

The present investigation allows the elimination of a few of these hypotheses. Thus, the light cells are not mere tangential sections of follicular cells, since they could be readily distinguished by the absence of colloid droplets after thyrotrophic hormone injection. Similarly, the hypothesis that light cells are follicular cells beginning mitosis (21) may be abandoned since, unlike dividing follicular cells, light cells are not in contact with the lumen, often show a hazy outline when in groups, and do not contain colloid droplets.

The position of light cells within the basement membrane of follicles makes it possible to eliminate several other hypotheses. Thus, at one time we wondered whether the groups of light cells were small ganglia. However, the typical ganglionic cells found on occasion in the thyroid gland (Fig. 9) were always in interfollicular locations. When it became clear that light cells were always intrafollicular (19), this position was felt to be a decisive argument against their neural nature (30). Since ultimobranchial bodies also had an interfollicular position and so did parathyroid cells whenever

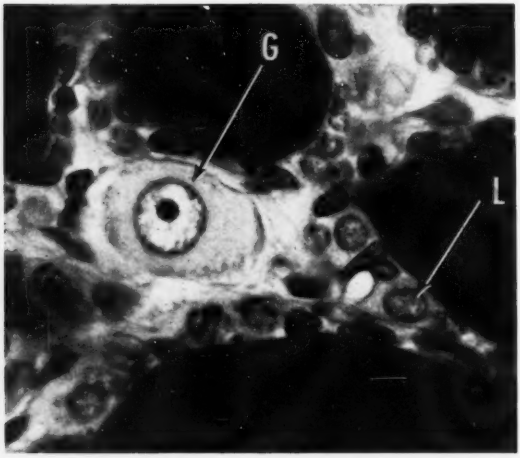

FIG. 9. Ganglionic cell in the thyroid of a hypophysectomized rat. Periodic acid-Schiff and hematoxylin. ×1000. The large ganglionic cell located in an interfollicular position differs from the light cells (L) by a round, central nucleolus, a uniform nucleus, and a cytoplasm with darkly staining material at the periphery.

they were seen within thyroid tissue, light cells may be considered to be neither remnants of an embryonic structure (8, 12), nor parathyroid cells (32). Incidentally the spatial relationship believed to exist between ultimobranchial bodies and light cells (12) was not observed in the rat (Table 3, Fig. 8).

The hypotheses implying a genetic relationship between follicular cells and light cells (9, 26, 36, 37) are in agreement with the epithelial nature of both cell types. However, follicular cells are *typical* epithelial cells, as shown by their close juxtaposition to one another, their separation from connective tissue and blood vessels by means of a basement membrane, and their orientation towards the follicular lumen, with terminal bars framing the cell apex. Light cells share some of these epithelial attributes, since they are enclosed with the follicular cells within a basement membrane; but they are not in contact with the lumen and lack terminal bars. The differences between the two cell types favor the concept of two distinct cell lines. Indeed they both show mitosis and therefore are able to reproduce themselves, and they are both capable of giving rise to characteristic tumors (2, 17, 18). Furthermore, since the proportion of light cells to follicular cells remains the same at all ages (Table 6) and since the number of cells in the thyroid gland keeps on rising with age (M. Enesco, unpublished), both follicular and light cells must be increasing at the same rate throughout life.

However, the distinction between the two cell types does not exclude the

possibility of a common origin. For instance one or several follicular cells might migrate or be displaced away from the epithelial layer, withdraw from the colloid and then lose some of the epithelial attributes, e.g. terminal bars. They might thus become light cells. The absence of colloid droplets in light cells may be interpreted in either one of two ways. The first possibility arises from the fact that light cells are separated from the colloid. The droplets are histochemically identical with the material in the lumen (28) and it has been suggested that they may be portions of colloid taken up from the lumen by the follicular cells through pinocytosis (20, 35). Since light cells are not in contact with the colloid, they cannot pinocytose colloid and, thus, remain free of droplets. The alternate possibility is that the droplets are a secretion product of the cell to be deposited in the lumen, that is, a cellular precursor of thyroglobulin (6, 7), in which case light cells would differ from follicular cells by the absence of such type of secretory activity.

In conclusion, the present data indicate that there are two morphologically distinct types of epithelial cells in the thyroid gland of the rat: the *follicular cells*, which are in great majority (usually over 95%) and a few *light cells*. Follicular cells are characterized by their polarization, contact with the colloid, and the ability to yield colloid droplets on injection of thyrotrophic hormone. Light cells lack these properties.

REFERENCES

1. Altmann, H. W.: *Beitr. Pathol. Anat.* **104:** 420. 1940.
2. Axelrad, A. A. and C. P. Leblond: *Cancer* **8:** 339. 1955.
3. Baber, E. C.: *Proc. Roy. Soc. London* **24:** 240. 1876.
4. Cowdry, E. B.: A Textbook of Histology. Lea & Febiger, Philadelphia. 1950.
5. Del Conte, E. and J. F. E. Vasena: *Ann. Endocrin.* **13:** 910. 1952.
6. Dempsey, E. W. and R. R. Peterson: *Endocrinology* **56:** 46. 1955.
7. De Robertis, E.: *Anat. Rec.* **84:** 125. 1942.
8. Dumont, L.: *Ann. Endocrin.* **17:** 700. 1956.
9. Florentin, P.: *Ann. Anat. path.* **6:** 1027. 1929.
10. Garden, H. S. D.: A Student's Histology. E. & S. Livingstone Ltd., Edinburgh and London. 1957.
11. Ghosh, A. C.: Thesis, McGill University, Montreal (1955).
12. Godwin, M. C.: Am. J. Anat. **60:** 299. 1937.
13. Greep, R. O.: Histology. The Blakiston Company Inc., New York and Toronto. 1954.
14. Ham, A. W.: Histology. 3rd Ed. J. B. Lippincott Co., Philadelphia and Montreal. 1957.
15. Heidenhain, M.: Quoted by Ludwig (21).
16. Hurthle, K.: Quoted by Ludwig (21).
17. Isler, H.: *J. Nat. Cancer Inst.* **23:** 675. 1959.
18. Isler, H., C. P. Leblond and A. A. Axelrad: *J. Nat. Cancer Inst.* **21:** 1065. 1958.
19. Isler, H., B. Thompson, R. Tonkin and S. K. Sarkar: *Anat. Rec.* **136:** 338 (1960).
20. Junqueira, L. C.: *Endocrinology* **40:** 286. 1947.
21. Ludwig, K. S.: *Acta Anat.* **19:** 28. 1953.

22. Ludwig, K. S.: *Acta Anat.* **20:** 1. 1954.
23. Maximow, A. A. and W. Bloom. A Textbook of Histology. W. B. Saunders Co., Philadelphia and London. 1957.
24. Nonidez, J. F.: *Am. J. Anat.* **49:** 479. 1932.
25. Pages, A.: *Biol. Méd.* **45:** 414. 1956.
26. Ponse, K.: L'Histophysiologie thyroidienne. Réunion des endocrinologistes de langue francaise, Paris, 2–3 julliet 1951. Masson, éditeur.
27. Saito, K. and K. Shibata: *Endocrin. Japon.* **4:** 253. 1957.
28. Sarkar, S. K. and H. Isler: Unpublished data.
29. Sugiyama, S.: *Anat. Rec.* **120:** 363. 1954.
30. Sunder-Plassmann, P.: *Deutsch Z. chir.* **253:** 210. 1939.
31. Takagi, K.: *Folia anat. Jap.* **1:** 69. 1922–23.
32. Tschassownikow, S. Quoted by Ludwig (21).
33. Van Dyke, J. H.: *Anat. Rec.* **88:** 369. 1944.
34. Van Dyke, J. H.: *Am. J. Anat.* **76:** 201. 1945.
35. Williams, R. G.: *Am. J. Anat.* **62:** 1. 1937.
36. Wolfer, A.: *Arch. klin. Chir.* **29:** 1. 1883.
37. Zechel, G.: *Surg. Gynec. Obstet.* **54:** 1. 1932.

RADIOIODINE EQUILIBRIUM STUDIES OF THYROID AND BLOOD

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ABSTRACT

A method of isotope equilibrium has been used to study iodinated amino acids of thyroid and blood in the rat. Iodide, monoiodotyrosine, diiodotyrosine, thyroxine and 3′,3,5-triiodothyronine were found to comprise 13%, 20%, 46%, 18%, and 3% of the thyroidal iodine respectively. Thyroxine accounted for approximately 80% of the blood iodine, triiodothyronine 3.5% and the iodinated tyrosines from zero to 4%. These figures are consistent with the hypothesis than in the rat, thyroxine and triiodothyronine contribute approximately half each of the total physiological activity of the thyroid hormones.

EARLY studies on the iodine content of the iodinated amino acids in the thyroid gland indicated that about 30% of it represented thyroxine[2] and the rest represented diiodotyrosine (1, 2, 3). Since that time, the discovery of monoiodotyrosine and 3,5,3′-triiodothyronine in the thyroid (4, 5) has called for a reassessment of these values. The thyroxine iodine has recently been estimated at between 20 and 30%, while monoiodotyrosine iodine accounts for 10 to 15% of thyroidal iodine (6). So far no estimate of the contribution of triiodothyronine to thyroidal iodine content has been made.

It was thought until recently that over 90% of the blood iodine was represented by thyroxine (7, 8, 9). However, two separate studies of the iodinated constituents of blood indicate that up to 50% of the blood iodine in normal individuals represents mono- and di-iodotyrosine (10, 11, 12, 13). The kinetics of iodotyrosine metabolism are such as to make their presence in the blood of normal animals somewhat unlikely. Furthermore, the analyses of the extremely small amounts of iodoamino acids of blood are difficult.

The present study was undertaken to re-evaluate these questions by the use of a technique of isotope equilibrium. In addition, it was hoped that correlation of thyroid and serum iodoamino acid contents with their turnover time in blood might make it possible to determine whether the iodoamino acids of blood are derived exclusively from the thyroid.

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[2] The following abbreviations are used in this paper: T_4 = thyroxine; T_3 = 3,5,3′-triiodothyronine; DIT = 3,5-diiodotyrosine and MIT = 3-monoiodotyrosine.

The method of isotope equilibrium has been used previously in a study of the thyroidal response to different diets (14) and different doses of stable iodine (15). If a labelled amount of iodine is given every day to an animal and the rate of turnover of iodine in the compartment with the slowest turnover is known, the time necessary to reach equilibrium can be calculated. At equilibrium radioactivity may be directly translated into mass units.

METHODS

Animals and Diets

Sprague Dawley male rats of 120–150 grams were used. All animals were placed on a low iodine diet, previously described and estimated to provide about 1 μg. I/day (16), for varying periods of time before and continuing throughout the experiment. Supplementary iodine was given as I^{131} labelled KI. In all experiments 10 μg. I^- as KI was given once daily by subcutaneous injection. The stock solution contained 2 μc. I^{131} per 10 μg. I^{127} on the first day of injection in Exp. 1 and 0.5 μc. per 10 μg. I^{127} on the first day in Exps. 2 and 3. Hence the animals received a different amount of I^{131} each day, but at the end of the experiment, radioactive assay alone revealed I^{127} values by comparison with the stock solution.

To determine how closely the experimental periods approached equilibrium, a group of rats was injected daily with 10 μg. I^{127} alone for 14 days and then received a single dose of 4.5 μc. of I^{131}. The daily injections of I^{127} were continued and the radioactivity in the thyroids of the animals was determined at frequent intervals for a week. External counting of unanesthetized animals was done with a well-shielded scintillation counter. Sufficient counts were taken to give a probable error of counting of $<5\%$ except for the last day of the experiment when the probable error was $<8\%$.

Chemical Procedures

Radioactive assay of whole thyroids and sera was done in a scintillation well counter with standardized geometry. Thyroid glands were hydrolyzed with pancreatin for 24 hours in Tris buffer, pH 8.4. Separation of iodinated amino acids from the thyroid hydrolysates was done with ascending paper chromatography using n-butanol, dioxane, $2N \cdot NH_4OH$, (4:1:5)(BDA) and n-butanol equilibrated with 2N-acetic acid. Carrier amino acids and iodide were chromatographed on the same paper and developed with ninhydrin and $PdCl_2$. Radioactivity was assayed with a recording rate meter calibrated so that areas under the counting curve were linearly proportional to the amount of radioactivity. For low activity spots (triiodothyronine in particular) the chromatographic strips were cut into segments corresponding with the location of the carrier amino acids and counted in a well type scintillation counter. Iodinated compounds in serum were separated by chromatography on Dowex 1×2, 200–400 mesh using acetate buffers as previously described (17). The combined thyroxine and triiodothyronine eluate was lyophylized and chromatographed in BDA.

Iodine analyses were done by modification of the Zak procedure (18) by the Boston Medical Laboratory.

RESULTS

The turnover rate of iodine in the thyroids of rats under the exact circumstances used in the subsequent work was checked with a single dose of I^{131}. In Figure 1 are shown the results of 4 animals. A turnover

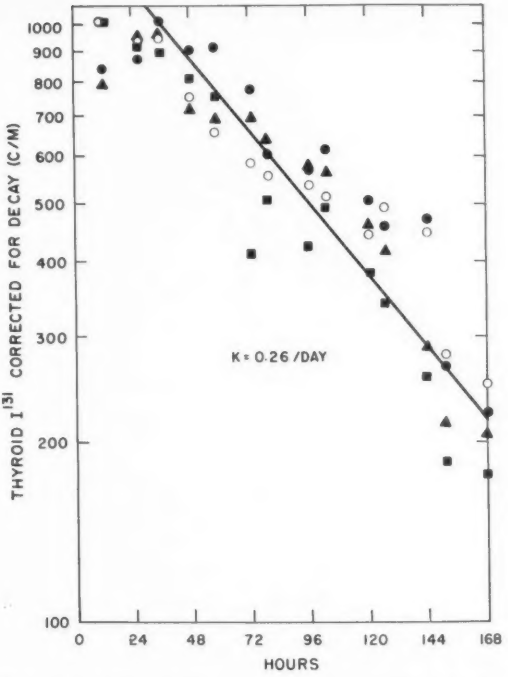

FIG. 1. Loss of I^{131} from rat thyroids after a single tracer dose of I^{131}. Different symbols represent different animals. Counts are corrected for physical decay of the isotopes.

rate of 26%/day was found from the single exponential decay of thyroidal I^{131}. It can be shown that 97% of equilibrium[3] will be obtained after 11.7 days under these circumstances.

Table 1 shows the general data from 3 separate experiments. The radiation doses were calculated from the maximum uptake of a single tracer dose of I^{131} in animals on the low iodine diet and receiving 10 μg. I per day, from the thyroid decay rate calculated from Figure 1 (26%/day), from the known thyroid weights (average 13 mg) and from the dose of

TABLE 1. TREATMENT OF EXPERIMENTAL GROUPS

Group	Number of animals	Time on diet (days)	Time on I^{131} (days)	Radiation to thyroid (rads)
1	6	20	14	4040
2	9	35	21	970
3	8†	27‡	25	1020

† A separate group of 8 animals treated identically were used for thyroid iodine analyses.
‡ These animals received 10 μg. iodine per day for 2 days prior to I^{131}.

[3] Equilibrium is defined as that state wherein all the iodinated materials in the animal have identical specific activities.

TABLE 2. CALCULATED AND EXPERIMENTAL VALUES FOR THYROID AND SERUM IODINE

Group	Thyroid iodine (calculated) (μg.)	Thyroid iodine (analyzed) (μg.)	Serum PBI† (calculated) (μg.%)	Serum PBI (analyzed) (μg.%)	Serum iodide (calculated) (μg.%)	Serum iodide (analyzed) (μg.%)
1	7.6		6.0			
2	7.5		4.0			
3	8.5	7.4	4.5	3.3	2.0	2.4

† PBI = protein bound iodine.

I^{131} administered. It was necessary to calculate the radiation from each day's dose of I^{131} since each varied in radioactivity and in length of stay in the animal. The sum of the daily doses at time of sacrifice is listed in Table 1. Experiment 3 was done in two parts and in one the thyroids were analyzed for I^{127} with results as shown in Table 2. In the other, the thyroids were counted and hydrolyzed. As can be seen, the agreement between analytical and calculated values for thyroidal iodine is fairly good. The calculated value was derived by a simple comparison of thyroidal radioactivity with an aliquot of the solution of iodine which had been administered to the animals.

The calculated and analytical values for blood iodine are also shown in Table 2. Again in Exp. 3, pooled sera from groups of animals treated identically were used for the calculated and analytical values since not enough blood was available for both analyses on each animal. Agreement between the values determined by chemical analysis and calculated from radioactivity is again reasonably good. No unidentified components were seen and the origin material on the chromatograms (presumably incompletely hydrolyzed thyroglobulin) usually contained 8% or less of the total radioactivity. Table 3 gives the results of analyses of hydrolyzed thyroids in 3 experiments and in Table 4 are the results of individual

TABLE 3. THYROIDAL I^{131} AFTER HYDROLYSIS EXPRESSED AS PER CENT OF TOTAL I^{131}

Group	I^-	MIT	DIT	T_4	T_3
1	8.9	30.5	42.4	16.4	1.8
2	11.6	19.2	46.4	19.6	3.2
3	13.0	20.1	45.9	18.0	3.0

TABLE 4. I^{131} IN FRACTIONS FROM INDIVIDUAL RAT THYROID HYDROLYSATES IN GROUP 3 EXPRESSED AS PER CENT TOTAL I^{131}

Rat No.	I^-	MIT	DIT	T_4	T_3
1	12.4	18.6	48.0	18.2	2.8
2	12.7	23.0	43.8	17.3	3.2
3	13.1	20.1	44.4	19.0	3.4
4	14.0	20.0	45.3	17.8	2.9
5	10.4	22.3	46.3	18.2	2.8
6	12.0	18.8	48.4	18.4	2.6

TABLE 5. SERUM I^{131} IN VARIOUS FRACTIONS EXPRESSED AS PER CENT OF TOTAL I^{131}

Group	T_4	T_3	MIT+DIT
2	94.5	4.2	1.3
3	92.2	4.4	3.4

analyses. Although equilibrium should be reached after 12 days of I^{131} injections, there is nevertheless an unexplained systematic difference in distribution of iodoamino acids between the 14- and 21-day studies. Twenty-one and 25 days give virtually identical results. The values in the tables represent per cent of total thyroidal iodine. If thyroidal organic iodine (the iodide is thereby neglected) is partitioned among these amino acids and the figures from Group 3 are used, the molar ratios of MIT, DIT, T_4 and T_3 respectively are 9.3; 15.0; 5.3 and 1.0.

Analyses of serum were done on groups 2 and 3 and the results are shown in Table 5. It may be seen that T_4 accounts for most of the serum iodine although T_3 is unequivocally present. In Group 2, only 4 of the 9 sera analyzed showed any radioactivity corresponding to either MIT or DIT. In Group 3, two analyses on pooled sera (5 animals each) gave radioactivity in the combined MIT and DIT fractions amounting to 3.6% and 3.2% of the total I^{131}.

DISCUSSION

There are certain advantages and several problems inherent in the use of isotope equilibrium for estimation of quantities of stable isotopes by radioactive measurements alone. One problem is that to obtain maximum sensitivity, the amount of radioactivity that must be administered may produce substantial radiation. As Table 1 shows, in Groups 2 and 3 the radiation (over the 3-week period) was approximately 1,000 rads. It is unlikely that this will produce physiological effects within that period of time. For thyroglobulin release, not only is more radiation required (over 3,000 rads) but it must be delivered in a short period of time (two or three days) (19, 20). Hence, we may tentatively conclude that radiation has not played a significant role in the present findings. The other problem associated with the use of isotope equilibrium is the proper estimation of the time necessary to attain equilibrium. Preliminary estimation of the turnover rate in the slowest compartment permits calculation of the time required to come as close to equilibrium is desired. As noted above it may be calculated that after 12 days, 97% of equilibrium should have been reached. This figure means that 97% of these animals' iodine was derived from the labeled iodine injected during the experimental period. However, as may be seen in Table 3, the MIT content is higher and the DIT and T_4 content is lower in Group 1 than in Groups 2 or 3. Since it has been commonly assumed that MIT is a precursor of DIT which in turn is a precursor of T_4, this might suggest that complete equilibrium had not

been obtained after 14 days. Incomplete mixing of thyroidal iodine resulting in more rapid secretion of radioactive than stable iodine could produce a similar picture (21). In this case a pool of iodine turning over more slowly in the thyroid would require a longer period of time for equilibration than would be estimated from tracer kinetics. Morel and Simon (22) found that somewhat over 80% of equilibrium was reached after 21 days with a diet containing 10 μg.I^-/day.

Since there is reasonably good agreement between iodine contents calculated from I^{131} data and estimated analytically (Tables 1 and 2), incomplete mixing or a separate pool of iodine in the thyroid turning over very slowly appear to be of minor significance. The figures for serum PBI are slightly higher than some values reported for the rat but were probably a result of the generous iodine supplement (23, 16).

The values for thyroidal iodoamino acids are similar to those reported by Roche and Michel (24) and Taurog *et al.* (6). The thyroxine value is lower than many have found although not lower than that found by Taurog *et al.* Roche and Michel (24) and Wolff and Chaikoff (25) found that T_4 iodine accounted for 0.25 to 0.34 of the total thyroidal iodine. Subsequent experiments to be reported later have shown that both thyroxine and triiodothyronine are partially deiodinated during hydrolysis with pancreatin. T_3 has not, however, been formed from T_4 during hydrolysis. Neither MIT nor DIT has been deiodinated appreciably during hydrolysis. It is likely therefore that the iodide is derived from T_4 and T_3. It has been shown (26) that unhydrolyzed rat thyroids only contain about 1% of inorganic iodine.

The results in blood are of interest and confirm the older views that T_4 is the major constituent of organic blood iodine (7, 8). These experiments have not revealed the presence of significant amounts of iodotyrosines in the blood of the rat. The value of T_3 in blood has been estimated to be about 0.14 μg.% in man (27). In the rat, Feuer (28) has found the level of triiodothyronine in serum to vary from 0.12 to 1.90 μg.%. The averages of the figures in Tables 2 and 4 give a value for stable T_3 in rat serum of 0.2 μg.%.

Since a deiodinase for T_4 and T_3 is lacking in the thyroid (29), it seems reasonable to assume that they will be secreted from the thyroid into the blood in the same proportion as they occur in the gland. It is, however, possible that partial hydrolysis of thyroglobulin with release of T_4 alone, followed by resynthesis, may occur. In this situation, the accumulation of thyroglobulin enriched in T_3 would be prevented if Feuer's suggestion that T_3 gives rise to T_4 did in fact take place. If this occurred, the ratio of secreted T_4:T_3 would differ from the relative concentrations of these amino acids in the thyroid. To assess these possibilities and the further possibility that T_3 in serum might be derived peripherally from the partial deiodination of T_4, the relative concentrations of T_4 and T_3 in thyroid and blood were compared. The steady state concentration of a compound in a

TABLE 6. CALCULATED AND EXPERIMENTAL VALUES FOR THYROXINE AND TRIIODOTHYRONINE IN THYROID AND SERUM

	Thyroxine	Triiodothyronine
Concentration in Thyroid	1.0*	0.17
Volume of distribution†	0.13/body wt	0.26/body wt
Degradation rate†	0.92/day	1.7/day
Calculated concentration in blood	1.0*	0.047
Concentration in blood found	1.0*	0.048

$$T_3(\text{calc.}) = \frac{R_dT_4}{R_dT_3} \cdot \frac{V_DT_4}{V_DT_3} \cdot \frac{T_eT_3}{T_eT_4}$$

where R_d = degradation rate
V_D = volume of distribution
T_e = thyroid concentration

* Normalized to thyroxine = 1. Thyroxine and triiodothyronine concentrations in thyroid and serum of animals from Group 3.

† Data of Feldman (30).

pool is a function of its rate of secretion into the pool, its rate of loss from the pool and the pool size.

Fortunately data are available on the pool sizes and the rates of degradation of T_4 and T_3 in the rat (30, 31). These figures are in good agreement and those of Feldman (30) are shown with calculated and observed values for T_4 and T_3 in blood in Table 6. The assumptions used for calculating the relative T_3 level in blood are:

1. All T_3 in blood is derived from T_3 in thyroid.
2. T_3 is secreted from the thyroid in the same ratio to T_4 as they occur in the thyroid.
3. The assumptions implicit in the calculations of pool size and degradation rate.

Although the precise agreement of calculated and analytical values is undoubtedly fortuitous, these findings indicate that thyroxine and triiodothyronine are secreted by the thyroid in the same ratio as they are present in the gland. The agreement also suggests that no significant amount of triiodothyronine in blood is derived from thyroxine as proposed by Pitt-Rivers *et al.* (32) but not confirmed by Lassiter and Stanbury (33) nor Dumont *et al.* (34).

The blood iodine data also indicate the physiological importance of triiodothyronine, at least in the rat. Since in this animal triiodothyronine is 4 to 5 times as potent as thyroxine in the goiter prevention test and in increasing oxygen consumption, the data in Table 5 suggest that about 40% of the total metabolic effect of thyroid hormone is due to triiodothyronine (see Barker (35) and Stasilli *et al.* (36)).

REFERENCES

1. HARINGTON, C. R. AND S. S. RANDALL: *Biochem. J.* **23**: 373. 1929.
2. GUTMAN, A. B., E. M. BENEDICT, B. BAXTER AND W. W. PALMER: *J. Biol. Chem.* **97**: 317. 1932.
3. CAVETT, J. W., C. O. RICE AND J. F. MCCLENDON: *J. Biol. Chem.* **110**: 673. 1935.

4. Fink, K. and R. M. Fink: *Science* **108:** 358. 1948.
5. Gross, J. and R. Pitt-Rivers: *Biochem. J.* **53:** 645. 1953.
6. Taurog, A., W. Tong and I. L. Chaikoff: *J. Biol. Chem.* **184:** 83. 1950.
7. Taurog, A. and I. L. Chaikoff: *J. Biol. Chem.* **176:** 639. 1948.
8. Laidlaw, J. C.: *Nature* **164:** 927. 1949.
9. Rosenberg, I. N.: *J. Clin. Invest.* **30:** 1. 1951.
10. Werner, S. C. and R. J. Block: *Nature* **183:** 406. 1959.
11. Block, R. J., S. C. Werner, R. H. Mandl, U. U. Row and I. Radichevitch: *Arch. Biochem. Biophys.* **88:** 98. 1960.
12. Whitehead, J. K. and D. Beale: *Clin. Chim. Acta* **4:** 710. 1959.
13. Beale, D. and J. K. Whitehead: *Clin. Chim. Acta* **5:** 150. 1960.
14. Van Middlesworth, L.: *Endocrinology* **58:** 235. 1956.
15. Simon, C. and F. Morel: *C. R. Soc. Biol. Paris* **151:** 1311. 1957.
16. Money, W. L., J. E. Rall and R. W. Rawson: *J. Clin. Endocrinol. & Metab.* **12:** 1495. 1952.
17. Galton, V. A. and R. Pitt-Rivers: *Biochem. J.* **72:** 310. 1959.
18. Zak, B., H. H. Willard, G. B. Myers and A. J. Boyle: *Anal. Chem.* **24:** 1345. 1952.
19. Tong, W., A. Taurog and I. L. Chaikoff: *J. Biol. Chem.* **195:** 407. 1952.
20. Robbins, J., J. E. Rall, D. V. Becker and R. W. Rawson: *J. Clin. Endocrinol. & Metab.* **12:** 586. 1952.
21. Triantaphyllidis, E.: *Arch. Sci. Physiol.* **12:** 245. 1958.
22. Morel, F. and C. Simon: *C. R. Soc. Biol. Paris* **151:** 1106. 1957.
23. Taurog, A. and I. L. Chaikoff: *J. Biol. Chem.* **165:** 217. 1946.
24. Roche, J. and R. Michel: *Biochim. Biophys. Acta* **1:** 335. 1947.
25. Wolff, J. and I. L. Chaikoff: *Endocrinology* **41:** 295. 1947.
26. Taurog, A., W. Tong and I. L. Chaikoff: *J. Biol. Chem.* **191:** 677. 1951.
27. Pind, K.: *Acta Endocrinol.* **26:** 263. 1957.
28. Feuer, G.: *Biochem. J.* **73:** 349. 1959.
29. Roche, J., R. Michel, O. Michel and S. Lissitzky: *Biochim. Biophys. Acta* **9:** 161. 1952.
30. Feldman, J. D.: *Amer. J. Physiol.* **188:** 30. 1957.
31. Maclagan, N. F. and J. H. Wilkinson: *J. Physiol.* **125:** 405. 1954.
32. Pitt-Rivers, R., J. B. Stanbury and B. Rapp: *J. Clin. Endocrinol.* **15:** 616. 1955.
33. Lassiter, W. B. and J. B. Stanbury: *J. Clin. Endocrinol. & Metab.* **18:** 903. 1958.
34. Dumont, J., A. M. Ermans and H. Galperin: *C. R. Soc. Biol. Paris* **153:** 360. 1959.
35. Barker, S. B.: *Ann. Rev. Physiol.* **17:** 417. 1955.
36. Stasilli, N. R., R. L. Kroc and R. I. Meltzer: *Endocrinology* **64:** 62. 1959.

FOLLICLE-STIMULATING HORMONE (FSH) AND INTERSTITIAL-CELL-STIMULATING HORMONE (ICSH) IN PITUITARY AND PLASMA OF INTACT AND OVARIECTOMIZED PROTEIN-DEFICIENT RATS[1,2]

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ABSTRACT

Absence of dietary protein in female rats caused a moderate increase in hypophyseal FSH content, whether expresssed per anterior pituitary or per milligram of tissue. The ICSH content in the hypophysis was unchanged from control levels per pituitary gland, though increased per milligram of tissue. Ovariectomy of protein-deficient rats further elevated pituitary content of both gonadotrophins, FSH and ICSH, raised the circulating levels of FSH and was accompanied by typical castration changes in the hypophysis. These morphological and physiological changes show that the pituitary gland, even in the complete absence of dietary protein, retained its capacity to respond to stimulus with increased synthesis and release of gonadotrophins.

THE detrimental effects of protein deprivation on the reproductive system are well known. Absence or low levels of dietary protein in rats has been shown to result in marked atrophy of ovaries and accessory organs with early loss of estrous cycles (2, 3). Follicular growth was arrested and interstitial cells closely resembled the "wheel cells" of hypophysectomy (3), suggesting some disturbance in hypophyseal-ovarian function: absence of dietary protein interfered either with the hypophyseal production and release of gonad-stimulating hormones, or with the responsiveness of the ovaries to these hormones. As previously reported (3), protein-deficient rats responded to exogenous hypophyseal gonadotrophins at the same dosage levels as hypophysectomized rats of comparable age and weight, suggesting that no impairment of ovarian sensitivity was present. In the present effort to elucidate the role of the pituitary in protein de-

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ficiency, bioassays for gonadotrophic hormone content have been conducted of the pituitaries of such deficient rats. These assays have been supplemented by cytological examination of the pituitaries and by bioassays of the blood plasma to obtain information regarding pituitary function. As a further means for estimating the capacity of the pituitary to respond physiologically to stimulus, protein-deficient rats were ovariectomized to determine whether the characteristic responses to gonadectomy, i.e., increased gonadotrophic content of pituitary and of plasma accompanied by castration changes in the hypophysis (4, 5), could be induced in the absence of dietary protein.

MATERIALS AND METHODS

Groups of adult female rats (Long-Evans strain) weighing approximately 200 gm. were maintained for 5 weeks on a purified diet free of protein but containing all other known dietary essentials.[3] Vaginal smears were taken daily; at the time of autopsy the animals had been anestrous for about one month and their reproductive organs were atrophic. A second series of rats was bilaterally ovariectomized and immediately placed on the protein-free diet for the same length of time. Groups of intact and of ovariectomized control rats were fed a purified diet containing 24% casein.[4] Additional intact control rats were maintained on the stock diet XIV and lettuce (3). Since bioassay results obtained with pituitaries and plasma of intact rats on the two complete diets were in close agreement, they have been combined in the Tables.

Methods for bioassay of anterior pituitary glands and plasma were those previously described by Wooten *et al.* (6) and Cozens and Nelson (7). Donor rats were exsanguinated by withdrawing blood from the aorta into heparinized syringes. Plasma was separated by centrifugation for 30 min. at 3200 rpm; it was then pooled and injected subcutaneously into immature female hypophysectomized rats the same day. Daily dosages varied from 2 to 10 cc., the higher levels being administered in doses of 2–3 cc. at 1–2 hour intervals. The recipients were autopsied 24 hours after the last injection. Uteri were freed from oviducts, drained of fluid and weighed. Ovaries were dissected, weighed, and fixed in Bouin's solution; they were then embedded in paraffin, serially sectioned, and stained with H & E.

Anterior pituitary glands removed immediately following exsanguination were weighed, ground and suspended in a known volume of isotonic saline containing 2% butanol. The suspensions were kept at 4° C until needed (usually for not more than 7 weeks). Serial dilutions were prepared, corresponding to total doses of 1/16 to 4 anterior lobes; injections were given subcutaneously, once daily for 4 days; autopsies were performed 24 hours after the last injection. Ovaries and uteri were weighed and the ovaries

[3] The protein-free diet contained sucrose 88%, hydrogenated cottonseed oil 8%, modified salts No. 4 4%. Crystalline vit./kg. of diet were: vit. B_{12} 0.05 mg., *d*-biotin 0.6 mg., 2-methyl-1,4-naphthoquinone 10 mg., thiamine HCl 10 mg., pyridoxine HCl 10 mg., pteroylglutamic acid 11 mg., riboflavin 20 mg., p-aminobenzoic acid 20 mg., niacin 40 mg., *d*-calcium pantothenate 100 mg., inositol 800 mg., choline chloride 1000 mg. All rats (control and protein-depleted) received weekly a fat-soluble vit. supplement of 800 U.S.P. units vit. A, 115 chick units vit. D, 6 mg. synthetic alpha-tocopherol and 650 mg. corn oil (Mazola).

[4] The complete diet contained alcohol-extracted casein 24%, sucrose 64%, hydrogenated cottonseed oil 8%, modified salts No. 4 4%, the vitamins listed above (except B_{12}) per 2 kg. of diet.

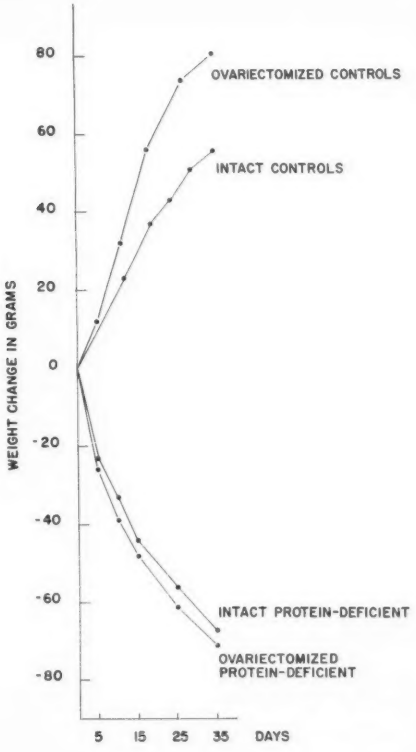


FIG. 1. Change in body weight (gm.) of intact and ovariectomized protein-deficient rats and of corresponding control animals.

prepared for histological examination. For histological study of the pituitaries, glands from each type of donor were fixed in Zenker-formol, embedded in nitrocellulose and stained with Mallory-Azan (8) and Periodic Acid-Schiff (PAS).

Estimates of the gonadotrophic content of donor pituitaries and plasma were based on the histological changes in the ovaries of recipients (9). The minimal effective dose (MED) for follicle-stimulating activity was that amount of anterior lobe suspension or plasma which induced resumption of follicular growth, as measured with an ocular micrometer, in 2 out of 3 recipient rats. The minimal effective dose for interstitial-cell-stimulating activity was the dose which was capable of inducing changes in the "deficient" ovarian interstitial tissue in 2 out of 3 recipients, as judged by the restoration of normal nuclear size, dispersal of chromatin material and the acquisition of eosinophilic cytoplasm. Groups of 2 to 4 assay rats were used for each dose level, and all critical dosage levels were repeated at least once.

RESULTS

The effects of protein deprivation on body weight are shown in Figure 1. Both intact and ovariectomized rats on protein-deficient diets lost from 55 to 70 gm., or approximately one-third of their initial body weight. Ovariectomized rats on the complete diet continued to gain weight at a rate slightly greater than that of their intact controls.

TABLE 1. GONADOTROPHIC CONTENT OF ANTERIOR PITUITARY GLANDS IN INTACT RATS

Diet fed to donors	Total dose		No. of recipients*	Ovaries			Uterus wt. (mg.)
	Glands No.	Wt. (mg.)		Wt. (mg.)	Follicles	Interstitial cells	
Complete	0	0	24	7.0	s†	deficient	15.2
	1/2	4.8	13	7.1	s	deficient	18.7
	1	9.6	12	9.9	s	part. repair	18.6
	2	19.2	14	11.7	s-sm	repair	22.8
	3	28.8	12	14.8	sm, m	repair	30.0
	4	38.4	6	29.5	m-l, CL (few)	repair	71.0e
Protein-free	0	0	21	7.3	s	deficient	14.9
	1/2	2.5	18	6.9	s	deficient	16.4
	1	4.9	18	8.9	s	part. repair	18.5
	2	9.8	14	17.2	sm, m	repair	52.7e
	3	14.7	3	32.9	m, ml	repair	93.1e
	4	19.8	4	67.8	m-l	repair	113.8e

* Female rats, hypophysectomized at 26–28 days of age and 17–19 days post-operative. They were maintained on stock diet I (3) plus lettuce twice weekly.

† Abbreviations: s = small, sm = small medium (380–500 μ), m = medium (550–650 μ), ml = medium large (700–750 μ), l = large (800–1000 μ); CL = corpora lutea and e = estrous uteri.

Pituitary bioassays

Intact donors.—Injection of one anterior pituitary from intact rats, whether fed the complete or the protein-free diet, induced partial repair of interstitial cells in the ovaries of recipient rats (Table 1). This dose level represents, therefore, the MED for ICSH activity; complete repair of interstitial tissue was attained at higher dosages.

Follicular development and ovarian and uterine weights remained unaffected when one pituitary from control donors was injected; two pituitaries initiated follicular growth in less than one-half of the injected rats, whereas three glands caused follicular growth in excess of the minimal histological response and also significantly increased ovarian and uterine weights. Therefore, the MED for FSH activity in pituitaries of intact controls was considered to be between two and three glands.

Anterior pituitaries from protein-deficient donors, likewise, caused no follicular growth when one gland was injected. However, two pituitaries produced a follicular response which exceeded minimal growth and which was comparable to that obtained from three control glands: the degree of response at higher dose levels also confirmed the higher FSH content in glands of rats on protein-free diets. Since pituitary weights were reduced in protein-deficient rats, hypophyseal content of both FSH and ICSH may be calculated to exceed that of control animals per milligram of anterior lobe tissue.

Ovariectomized donors.—Bioassays of anterior pituitaries from ovariectomized rats maintained on the complete diet showed the anticipated increase in gonadotrophic content (Table 2). Doses as small as $\frac{1}{8}$ gland caused partial repair of ovarian interstitial cells in most recipient rats

TABLE 2. GONADOTROPHIC CONTENT OF ANTERIOR PITUITARY GLANDS IN OVARIECTOMIZED RATS

Diet fed to donors	Total dose		No. of recipients*	Ovaries			Uterus wt. (mg.)
	Glands No.	Wt. (mg.)		Wt. (mg.)	Follicles	Interstitial cells	
Complete	0	0	16	6.6	s*	deficient	15.9
	1/16	0.6	11	8.6	s	deficient	17.0
	1/8	1.3	11	10.6	s	part. repair	17.1
	1/4	2.5	18	14.7	s-sm	repair	24.6
	1/2	5.1	17	34.0	m-l, CL	repair	67.5e
Protein-free	0	0	16	7.7	s	deficient	16.3
	1/16	0.4	13	8.7	s	deficient	16.6
	1/8	0.8	12	10.3	s	part. repair	17.0
	1/4	1.7	20	15.6	s-sm	repair	28.7
	1/2	3.3	20	41.4	m-l, CL	repair	69.8e

* See footnotes to Table 1.

higher doses fully restored normal nuclear pattern and cell size. Doses of $\frac{1}{4}$ gland initiated follicular growth in some assay rats, whereas $\frac{1}{2}$ gland gave a response exceeding minimal stimulation. At the latter dose, follicles of all sizes, corpora lutea and estrous uteri were observed. Similar bioassay results have been obtained for anterior pituitaries of rats ovariectomized and maintained on a stock diet of natural foodstuffs for one month (10).

Anterior lobes from ovariectomized, protein-deficient donors began to repair interstitial cells at doses of $\frac{1}{8}$ anterior pituitary and caused follicular growth at doses between $\frac{1}{4}$ and $\frac{1}{2}$ gland. No difference in gonadotrophin content could be detected within the dose range tested, between the pituitaries of ovariectomized rats maintained on complete or protein-free diets; qualitative and quantitative responses of the reproductive tracts of recipients were comparable at the same dose levels. From this it was concluded that the anterior pituitaries of ovariectomized rats deprived of dietary protein for 5 weeks contained as much FSH and ICSH as glands of ovariectomized rats on complete dietary regimen.

Plasma bioassays

Intact donors.—In agreement with previous reports (7), neither FSH nor ICSH activity was detected in the plasma of intact donors by the bioassay method used, even when total doses of 32–40 cc. were administered (Table 3).

Ovariectomized donors.—FSH activity could be detected in the plasma of ovariectomized rats on complete diets, a finding in agreement with previous reports (7). A total dose of 16 cc. of plasma stimulated follicular growth in the ovaries of approximately one-half of the recipient rats, whereas 24 cc. exceeded minimal follicular development; the MED, therefore, must lie between these two levels. Administration of 16 and 24 cc.

TABLE 3. GONADOTROPHIC POTENCY OF RAT PLASMA

Donors		Total dose (cc.)	No. of recipients*	Ovaries			Uterus wt. (mg.)
Type	Diet			Wt. (mg.)	Follicles	Interstitial cells	
Intact	Complete	0	10	7.6	s*	deficient	16.2
		32	10	8.3	s	deficient	18.0
	Protein-free	0	7	7.5	s	deficient	15.0
		32–40	5	7.4	s	deficient	16.1
Ovariectomized	Complete	0	12	6.5	s	deficient	17.2
		8	9	6.1	s	deficient	18.0
		16	12	7.1	s-sm	deficient	18.7
		24	7	9.3	sm, m	deficient	27.3
	Protein-free	0	16	6.4	s	deficient	16.3
		8	6	7.1	s	deficient	15.8
		16	10	11.6	sm, m	deficient	22.9
		24	7	15.8	m-ml	deficient	38.1

* See footnotes to Table 1.

of plasma from ovariectomized, protein-deficient animals resulted in more follicular stimulation and greater organ weights than was obtained with equivalent doses of plasma from ovariectomized rats on complete diets; the MED for FSH appeared to be slightly less than 16 cc. Therefore, an increase in circulating FSH activity following ovariectomy occurred in the complete absence of dietary protein; in fact the increase appeared to equal or even exceed that observed in ovariectomized rats maintained on the complete diet. No stimulation of interstitial cells (ICSH activity) was noted after injection of plasma from ovariectomized donors maintained either on complete or protein-free diets.

Pituitary cytology.

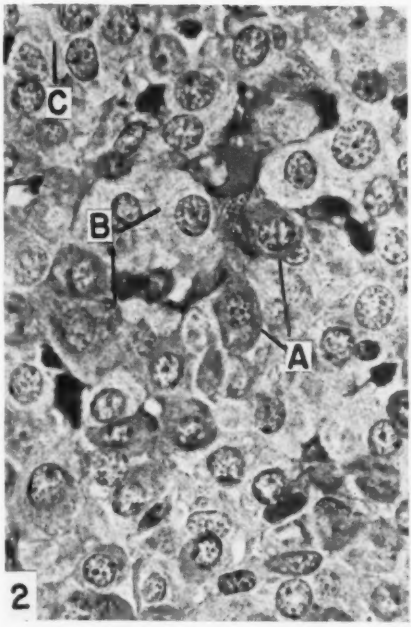
Certain distinguishing morphological characteristics could readily be noted in the pituitary glands of different types of donors, even though no detailed cell counts or measurements were made.

FIG. 2. Anterior pituitary of female rat maintained on the purified complete diet, showing normal appearance and distribution of acidophils (A), basophils (B) and chromophobes (C). (Mallory-Azan, ×800.)

FIG. 3. Anterior pituitary of female rat maintained on the protein-free diet for 5 weeks. Due to degranulation of acidophils and reduction in their nuclear and cell size the parenchyma assumed a very dense appearance. (Mallory-Azan, ×800.)

FIG. 4. Anterior pituitary of a rat ovariectomized and maintained on the purified complete diet for 5 weeks. Several enlarged basophils with prominent negative Golgi images and a "signet-ring" cell are shown. (Mallory-Azan, ×800.)

FIG. 5. Anterior pituitary of a rat ovariectomized and maintained on the protein-free diet for 5 weeks. Typical castration changes (cf. Fig. 4) are in evidence. (Mallory-Azan, ×800.)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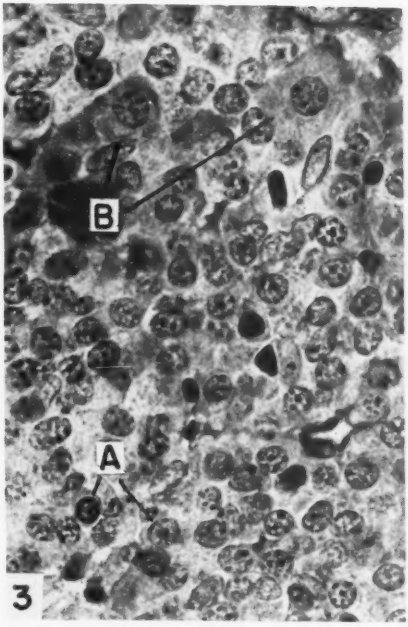
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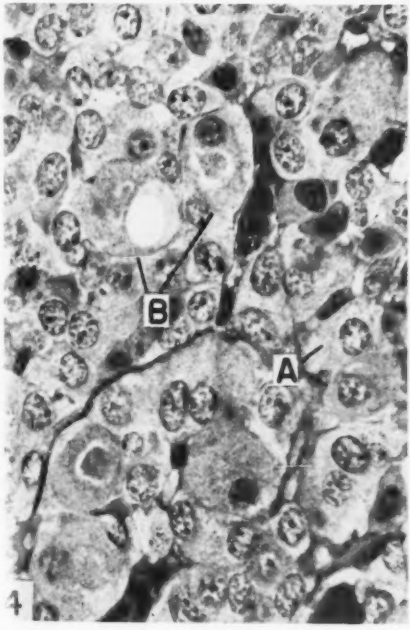
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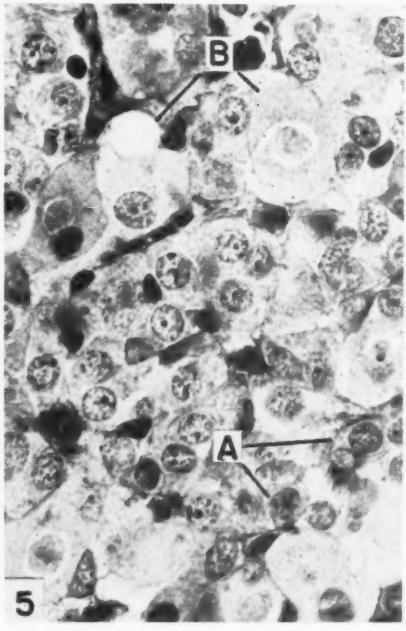
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Intact donors.—Anterior lobes of rats fed the complete diet were characterized by cells with ample cytoplasm and large, vesicular nuclei (Fig. 2). Comparable areas of pituitaries from protein-deficient rats contained many more cells per unit area, judged by the number of nuclei in any given microscopic field (Fig. 3), presumably due to reduced cell size. As previously reported, acidophils were decreased in number (11). Those remaining had deeply stained nuclei and a narrow rim of degranulated cytoplasm. Basophils were unchanged or slightly increased in number.

Ovariectomized donors.—Changes typical of castration were observed in basophils of anterior pituitaries from the ovariectomized control rats; the changes ranged from increase in cell number and size with enlargement of the Golgi apparatus to formation of "signet-ring" cells (Fig. 4). Similar changes were present in pituitary basophils of ovariectomized rats on protein-free diets (Fig. 5). Great numbers of enlarged basophils with prominent negative Golgi images were present throughout the gland. Regressive changes in acidophils previously noted in anterior lobes of intact protein-deficient animals were likewise present in glands of ovariectomized rats deprived of dietary protein.

DISCUSSION

The results of pituitary and plasma bioassays for the different types of experimental animals are compared in Table 4. They demonstrate that anterior pituitaries of intact female rats on protein-free diets contained as much ICSH and slightly more FSH than glands of intact control rats receiving nutritionally complete diets. Although the gonads of rats on protein-free diets were markedly subnormal, no cytological changes typical of castration were found in the pituitaries; at most, the number of basophils was slightly increased.

No previous reports have been found in the literature relative to the gonadotrophic content in pituitaries of female rats on protein-free diets. The results from inanition, which necessarily also limits protein intake, have been conflicting (12), possibly attributable to differences in experimental design and bioassay methods. Rinaldini (13) has reported that food

TABLE 4. GONADOTROPHIC CONTENT OF ANTERIOR PITUITARY AND PLASMA FROM INTACT AND OVARIECTOMIZED PROTEIN-DEFICIENT OR CONTROL RATS

Donors		Anterior pituitary		Plasma
		FSH MED	ICSH MED	FSH MED
Type	Diet	No. glands	No. glands	cc.
Intact	Complete	2–3	1	none detected
	Protein-free	1–2	1	none detected
Ovariectomized	Complete	1/4–1/2	1/8	$\geq$16
	Protein-free	1/4–1/2	1/8	$\leq$16

restriction caused a significant increase in total pituitary gonadotrophic content, as judged by the ovarian weight response in hypophysectomized assay rats. This increase in potency has been found to be closely correlated with definite cytochemical changes in the hypophysis (14).

There have been several reports concerning the effects of other dietary deficiencies on hypophyseal gonadotrophins. Vitamin B_6-deficiency for two months has been shown to increase markedly hypophyseal FSH content (6), as judged by criteria identical to those used in this paper; ICSH content was only slightly greater than that of control rat pituitaries. In contrast, anterior pituitaries of rats deprived of thiamine for seven weeks were markedly depleted of gonadotrophic hormones (15). No uniform results have been obtained with pituitary bioassays of vitamin E-deficient female rats (12). In this study, high levels of all vitamins were provided to insure adequate intake and to rule out the effects of other dietary deficiencies.

The findings of a slight increase in the pituitary content of FSH in intact rats on protein-free diets, without detectable rise in blood levels and accompanied by gonadal atrophy, are consistent with the hypothesis that protein deprivation results in failure of hormone release from the pituitary, probably associated with a reduced rate of hormone sythesis. Such an interpretation has been advanced previously to explain the effects of acute starvation (16). However, ovariectomy of protein-deficient rats greatly increased hypophyseal FSH and ICSH content, raised circulating levels of FSH and brought about castration changes in the hypophysis. Furthermore, the increases in pituitary and plasma gonadotrophic levels were equivalent to those obtained following ovariectomy of rats maintained on nutritionally complete diets. It appears, therefore, that the pituitary gland is able to respond to gonadectomy with an increase in the production and release of gonadotrophic hormones, even in the complete absence of dietary protein. The cytological appearance of the pituitary basophils is in keeping with this assumption.

It should be noted that protein deprivation, described here as increasing pituitary FSH in female rats, has been reported to reduce the content of the gonadotrophins in pituitaries of male rats (17, 18). Nevertheless, gonadectomy of young male rats on protein-free diets was found to increase hypophyseal gonadotrophic content, though only to levels prevailing in intact controls (17).

REFERENCES

1. Srebnik, H. H. and M. M. Nelson: *Anat. Rec.* **127:** 372. 1957.
2. Guilbert, H. R. and H. Goss: *J. Nutrit.* **5:** 251. 1932.
3. Srebnik, H. H., M. M. Nelson and M. E. Simpson: *Proc. Soc. Exper. Biol. & Med.* **99:** 57. 1958.
4. Severinghaus, A. E.: *Physiol. Rev.*, **17:** 556. 1937.
5. Finerty, J. C.: *Physiol. Rev.* **32:** 277. 1952.

6. Wooten, E., M. M. Nelson, M. E. Simpson and H. M. Evans: *Endocrinology* **56:** 59. 1955.
7. Cozens, D. A. and M. M. Nelson: *Proc. Soc. Exper. Biol. & Med.* **98:** 617. 1958.
8. Koneff, A. A.: *Stain Technology* **13:** 49. 1938.
9. Evans, H. M., M. E. Simpson, S. Tolksdorf and H. Jensen: *Endocrinology* **25:** 529. 1939.
10. Cozens, D. A. and M. M. Nelson: *Endocrinology*. In press.
11. Srebnik, H. H., M. M. Nelson and M. E. Simpson: *Proc. Soc. Exper. Biol. & Med.* **101:** 97. 1959.
12. Ershoff, B. H.: *Vitamins and Hormones* **10:** 79. 1952.
13. Rinaldini, L. M.: *J. Endocrinol.* **6:** 54. 1949.
14. Pearse, A. G. E. and L. M. Rinaldini: *Brit. J. Exper. Path.* **31:** 540. 1950.
15. Hundhausen, G.: *Arch. Exp. Path.* **192:** 670. 1939.
16. Maddock, W. O. and C. G. Heller: *Proc. Soc. Exper. Biol. & Med.* **66:** 595. 1947.
17. Leathem, J. H.: *Rec. Prog. Hormone Res.* **14:** 141. 1958.
18. Srebnik, H. H. and M. M. Nelson: *Anat. Rec.* **136:** 284. 1960.

THE ESTROGENIC PROPERTIES *IN VITRO* OF DIETHYLSTILBESTROL AND SUBSTANCES RELATED TO ESTRADIOL[1]

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ABSTRACT

A number of natural and synthetic estrogens and their structural analogs were tested for their effectiveness in stimulating or inhibiting the estrogen dependent pyridine nucleotide transhydrogenase of human placenta. Diethylstilbestrol is a potent stimulator of the enzyme system at low concentrations but is an inhibitor at concentrations above 3×10^{-6} M. The stilbestrol analog, 1,3 di-p-hydroxylphenyl propane, has only inhibitory effects on the system. Estrololactone, testololactone and Δ-1 testololactone neither stimulate nor inhibit the transhydrogenase. Estrone sulfate has stimulatory activity equal to estrone or estradiol whereas estradiol disulfate is inactive. It becomes active when the sulfates are removed by solvolysis. The thiol analogs of estradiol, with an −SH group in place of the −OH at carbon 17 are not dehydrogenated by estradiol-17β dehydrogenase nor can they stimulate transhydrogenase. They are potent inhibitors of estradiol-17β dehydrogenase, with the β thiol analog being more effective than the α thiol analog, but are only weak inhibitors of the transhydrogenase. Certain inferences are made regarding the structural requirements for activity in stimulating the transhydrogenase system.

THE estrogen-dependent pyridine nucleotide transhydrogenase of human placenta can be stimulated by a variety of estrogenic substances (1, 2) and the estrogenic stimulation can be inhibited competitively by a number of antiestrogenic compounds (3, 4). One finding of special interest was that diethylstilbestrol, which is a potent estrogen *in vivo* (5), was only weakly estrogenic when added alone to the transhydrogenase system and it decreased the rate of transhydrogenation when added in combination with estradiol (6). These experiments were carried out with the soluble proteins obtained by high speed centrifugation of a placental homogenate. With the development of methods for obtaining more purified transhydrogenase preparations, a reinvestigation of this phenomenon seemed desirable. This paper reports that at very low concentrations diethylstilbestrol is a potent activator of the estrogen dependent pyridine nucleotide transhydrogenase system, one with a molar effectiveness comparable to

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estradiol-17β. At higher concentrations it becomes an inhibitor. The investigation was extended to include a number of synthetic substances structurally related to the natural estrogens.

MATERIALS AND METHODS

Fresh human term placentas, obtained immediately after delivery, were homogenized and centrifuged as described previously (7). The material not sedimented by a force of 57,000×g for one hour was treated with ammonium sulfate and the fraction precipitating between 30 and 40% saturation with ammonium sulfate was used directly in some experiments or was further purified before use by the method of Hagerman and Villee (8). This procedure includes heat denaturation of contaminating protein and adsorption of the active protein to, and elution from, calcium phosphate gel.

Transhydrogenase activity was measured by coupling the enzyme with glucose-6-phosphate dehydrogenase as a TPNH generating system. Assays were carried out in a Beckman DU spectrophotometer at 23° C at a wavelength of 340 mμ in glass cuvettes with a 1 cm. light path. Each cuvette contained 200 micromoles of Tris buffer, pH 7.4, 10 micromoles Mg^{++}, 4×10^{-3} micromoles of Zn^{++}, 3.8 micromoles of glucose-6-phosphate, 0.4 Kornberg units of glucose-6-phosphate dehydrogenase, 0.05 micromoles of TPN, the enzyme preparation, and the steroid or other substance to be tested. The control cuvette did not contain the steroid but contained an equal volume of the solvent in which the steroid was dissolved. The final volume in each cuvette was 3.0 ml. Recordings were made until a stable baseline was obtained; then the reaction was initiated by the addition of 3.4 micromoles of DPN in 0.3 ml. of water. The optical density at 340 mμ was measured at 60 second intervals for a period of 11 or more minutes, during which the change in optical density with time was linear. To make comparisons between experiments using different enzyme preparations, the control rate for each experiment was normalized to 100.

Measurements of transhydrogenase activity by the reaction DPNH+APDPN→DPN+APDPNH, were carried out in a Beckman DU spectrophotometer at 23° C at a wavelength of 400 mμ, in glass cuvettes with a 1 cm. light path. Each cuvette contained 200 micromoles Tris buffer, pH 7.4, 1.3 micromoles DPNH, the steroid, enzyme and water to a final volume of 3.0 ml. The reaction was started by the addition of 1.5 micromoles of APDPN. Steroid was omitted from the control cuvette.

Estradiol dehydrogenase activity was assayed in a Beckman DU spectrophotometer at 23° C at a wavelength of 340 mμ in glass cuvettes with a 1 cm. light path. Each cuvette contained 200 micromoles of Tris buffer, pH 8.4, 10 micromoles of Mg^{++}, 25 mg. of serum albumin, enzyme, and the steroid dissolved in propylene glycol, all in a final volume of 3.0 ml. The control cuvette contained an equal volume of propylene glycol alone. The reaction was begun by adding 2.0 micromoles of DPN to all tubes and the reaction was followed for 15 minutes.

RESULTS

The effects of diethylstilbestrol, alone and in combination with estradiol on the activity of the transhydrogenase system are shown in Table I. Stilbestrol is an inhibitor when added in concentrations exceeding 3.7×10^{-7} M (1 μg./ml.) as reported previously (3). However, between 3.7×10^{-6} M and 3.7×10^{-7} M stilbestrol is an effective estrogen in this *in vitro* system with an activity comparable to that of estradiol-17β.

Estrololactone, testololactone and Δ-1-testololactone were tested at

TABLE 1. EFFECTS OF ESTRADIOL-17β AND DIETHYLSTILBESTROL ON THE ESTROGEN DEPENDENT PYRIDINE NUCLEOTIDE TRANSHYDROGENASE OF HUMAN PLACENTA

Estradiol concentration, M	Diethylstilbestrol concentration, M					
	0	3.7×10^{-7}	1.2×10^{-6}	3.7×10^{-6}	1.2×10^{-5}	1.2×10^{-4}
0	100	129	480	100	85	44
3.7×10^{-8}	133	176	520	180	155	60
3.7×10^{-7}	255	327	435	230	169	86
3.7×10^{-6}	370	390	415	222	163	106

Change in optical density at 340 μm per minute during the initial, linear phase of the reaction was calculated for each cuvette, then expressed as rate relative to the control, to which no hormone was added, set at 100. The absolute values of the reaction rates in the absence of added hormone of the enzyme preparations from different placentas used in the experiments summarized in tables 1, 2, 3, and 5 ranged from 0.030 to 0.100 optical density units per ten minutes. The values for estradiol alone are the means of eight experiments, the values for stilbestrol alone are the means of four experiments and the values for the various combinations of stilbestrol and estradiol are the means of two observations.

found to be without effect, over the concentration range from 10^{-4} M to 10^{-7} M, on the transhydrogenase system. They did not stimulate the system when added alone and they did not inhibit the system when added together with estradiol-17β. Dibenzyline was also tested and found to be without stimulatory or inhibitory activity.

Equilin and an analog of stilbestrol, 1,3 di-p-hydroxyphenyl propane had been tested previously in a system in which the transhydrogenase was coupled with isocitric dehydrogenase. When these compounds were re-examined with the estrogen dependent transhydrogenase coupled to glucose-6-phosphate dehydrogenase the results obtained confirm and extend the earlier observations. Table 2 shows that equilin is a very effective stimulator of the transhydrogenase system and that it does not appear to inhibit the stimulatory action of estradiol. When the two are added together at low concentrations, their stimulatory effects are additive. The stilbestrol analog had no stimulatory effect alone but was a potent inhibitor when added along with estradiol (Table 3). This confirms our previous findings using a different test system (3). Thus the analog differs from stilbestrol in having no estrogenic activity at low concentrations.

TABLE 2. EFFECTS OF EQUILIN AND ESTRADIOL-17β ON THE ESTROGEN DEPENDENT PYRIDINE NUCLEOTIDE TRANSHYDROGENASE OF HUMAN PLACENTA

Estradiol, M	Equilin, M			
	0	1.2×10^{-6}	1.2×10^{-5}	1.2×10^{-4}
0	100	225	346	362
3.7×10^{-8}	127	325	375	683
3.7×10^{-7}	227	345	390	427
3.7×10^{-6}	332	353	331	253

The change in optical density at 340 mμ per minute during the initial, linear part of the reaction was calculated for each cuvette, then expressed as rate relative to the control, to which no hormone was added, set at 100. The values are the means of two observations.

TABLE 3. EFFECTS OF ESTRADIOL-17β AND 1,3-DI-P-HYDROXYPHENYL PROPANE ON THE ESTROGEN DEPENDENT PYRIDINE NUCLEOTIDE TRANSHYDROGENASE OF HUMAN PLACENTA

Estradiol, M	1,3 di-p-hydroxyphenyl propane, M			
	0	1.5×10^{-6}	1.5×10^{-5}	1.5×10^{-4}
0	100	104	83	51
3.7×10^{-8}	125	76	93	64
3.7×10^{-7}	234	188	171	70
3.7×10^{-6}	276	235	181	121

The change in optical density at 340 mμ per minute during the initial, linear part of the reaction was calculated for each cuvette, then expressed as rate relative to the control, to which no hormone was added, set at 100. The values are the means of two observations.

A series of experiments with estrone sulfate revealed that it has essentially the same stimulatory effect on the transhydrogenase system as estrone or estradiol. In contrast, estradiol disulfate was completely without activity in the system; either as a stimulator when added alone or as an inhibitor when added with estradiol-17β. The estradiol disulfate was subjected to solvolysis by the method of Burstein and Lieberman (9) for 24 hours at room temperature. A sample of estradiol-17β was carried through the same procedure as a control. After solvolysis, samples were dissolved in methanol placed on Whatman #1 filter paper and chromatographed in a 2:1 ligroin: toluene/70% methanol system, together with standards of estradiol-17β and estradiol disulfate. The chromatograms were then dried and sprayed with ferrocyanide reagent. Estradiol disulfate does not react with this dye. The sample of estradiol disulfate which had undergone solvolysis did react with the dye and its Rk was identical with that of the standard estradiol-17β, which is evidence that it had been converted to estradiol. The product of solvolysis was tested in the coupled transhydrogenase system and now was an effective stimulator of the transhydrogenase system, as effective as pure estradiol-17β. Thus, although the hydroxyl at C 3 can be sulfated without loss of activity (as in estrone sulfate) sulfating the hydroxyl group at C 17 results in complete loss in activity. The activity can be restored by removing the ester sulfate.

The two thiol analogs of estradiol, with an $-$SH group in place of the $-$OH group at carbon 17 in the α and β configurations were tested as substrates and inhibitors of estradiol dehydrogenase and as stimulators and inhibitors of the estrogen dependent pyridine nucleotide transhydrogenase. Neither the α- nor the β-thiol was dehydrogenated by the placental estradiol dehydrogenase. The $-$SH group has a larger Van der Waals' radius than does an $-$OH group and this may prevent the substrate from fitting properly onto the active site of the enzyme; or the oxidation-reduction potential of the $-$SH group might be too low to permit its dehydrogenation by this enzyme. No comparable enzymatic dehydrogenation of a sulfhydryl group to a thioketone is known in mammalian metabolism. Both the 17β thiol and the 17α thiol compound inhibit the

TABLE 4. EFFECTS OF THE 17 THIOL ANALOGS OF ESTRADIOL ON ESTRADIOL-17β DEHYDROGENASE (DPN) OF HUMAN PLACENTA

Estradiol, M	Concentration of thiol analog, M							
	0	4.6×10^{-6}	9.2×10^{-6}	1.84×10^{-5}	4.6×10^{-5}	9.2×10^{-5}	1.38×10^{-4}	1.84×10^{-4}
1.95×10^{-5}	29	27	25	22	16	4	0	3
		11	3	13	5	0	6	6
4.86×10^{-5}	106	63	45	19	30	22	17	14
		47	24	18	22	0	5	8
9.72×10^{-5}	183	137	156	124	46	44	44	13
		115	70	16	5	0	0	2
1.46×10^{-4}	179	158	121	75	68	62	49	21
		125	81	48	5	9	19	7
1.95×10^{-4}	180	141	96	94	96	72	54	25
		132	76	78	90	49	23	17

The results are expressed as changes in optical density at 340 mμ for the initial 5 minutes times 10^3. The values given are the means of two or more observations. The values for the α analog are given above and the values for the β analog are given below in each row.

dehydrogenation of estradiol by estradiol dehydrogenase (Table 4). The 17β thiol compound is a more potent inhibitor of estradiol dehydrogenase than is the 17α thiol compound. The enzyme is believed to attack the alpha side of the steroid (10). Estradiol-17α is a potent inhibitor of estradiol-17β dehydrogenase (11) and the finding that the 17β thiol is a more potent inhibitor than the 17α thiol probably reflects a greater affinity of the enzyme for the β thiol compound than for the α thiol compound.

Since previous experiments had indicated a requirement for a β-hydroxy or a keto group at carbon 17 for estrogenic activity in the transhydrogenase system, the possible effect on this system of substituting a thiol group for the hydroxy group was of interest. Experiments revealed that neither the 17β thiol nor the 17α thiol could stimulate transhydrogenation, either in a system coupled with glucose-6-phosphate dehydrogenase as TPNH generator or in a system in which the transfer of hydrogen from DPNH to APDPN was measured. The thiol analogs were not as potent in inhibiting the transhydrogenase as they were in inhibiting the dehydrogenase (Table 5). When the molar ratio of thiol analog:estradiol was 10 there was

TABLE 5. EFFECTS OF ESTRADIOL-17β AND THE 17 THIOL ANALOGS OF ESTRADIOL ON THE ESTROGEN DEPENDENT PYRIDINE NUCLEOTIDE TRANSHYDROGENASE OF HUMAN PLACENTA

Conc. of thiol, M	Control	Estradiol 3.7×10^{-6} M alone	Estradiol 3.7×10^{-6} M plus estradiol-17β thiol	Estradiol 3.7×10^{-6} M plus estradiol-17α thiol
3.7×10^{-7}	100	213	217	220
3.7×10^{-6}	100	220	242	238
3.7×10^{-5}	100	220	237	235
5.5×10^{-5}	100	217	144	220
7.4×10^{-5}	100	212	108	172

The change in optical density at 340 mμ per minute during the initial, linear part of the reaction was calculated for each cuvette, then expressed as rate relative to the control, to which no hormone was added, set at 100. The values are the means of four observations.

no inhibition of transhydrogenase with either the β thiol or the α thiol. An inhibition with the β thiol analog was observed only when the molar ratio reached 15 and an inhibition with the α thiol analog was not observed until the molar ratio of analog:estradiol was greater than 20. In contrast a marked inhibition of the estradiol dehydrogenase reaction was observed with both β and α thiol analogs when the ratio of analog:estradiol was 1.

DISCUSSION

These investigations show that diethylstilbestrol is a potent stimulator of the placental estrogen dependent pyridine nucleotide transhydrogenase when added *in vitro* at low concentrations. The effect reaches a maximum at about 10^{-7} M and the substance becomes inhibitory at concentrations above 3×10^{-6} M. A remarkably similar phenomenon has been described by Mueller (12) in the effect of estradiol on phospholipid synthesis by segments of rat uterus incubated *in vitro*. At concentrations of 10^{-7} M estradiol is stimulatory but at higher concentrations it is an inhibitor. Thus this synthetic estrogen as well as the natural estrogens have an effect on the transhydrogenase system which parallels their effect *in vivo*. In contrast, the analog of stilbestrol, 1,3 di-p-hydroxyphenyl propane, which is an antiestrogen *in vivo* (13), has only inhibitory effects on the transhydrogenase system.

The complete ineffectiveness of estrololactone, testololactone and Δ-1 testololactone as either stimulators or inhibitors of the transhydrogenase system is further evidence of the structural requirement of the configuration around carbon 17 of the steroid molecule. The ineffectiveness of estradiol disulfate as a stimulator of transhydrogenase and its effectiveness following its solvolysis to free estradiol also emphasizes the structural requirement for a free hydroxy or keto group at carbon 17 for activity in the transhydrogenase system. The inability of the thiol analogs to serve as stimulators of transhydrogenase further points up the requirement for a hydroxy or keto group at carbon 17 for biological activity. The fact that both thiol analogs are so much more potent inhibitors of estradiol dehydrogenase than they are of the estrogen dependent pyridine nucleotide transhydrogenase is one more example of the different characteristics of these two enzymes (14).

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REFERENCES

1. Villee, C. A. and E. E. Gordon: *Bull. Soc. Chim. Belges* **65**: 186. 1956.
2. Hollander, V. P., H. Noland and N. Hollander: *J. Biol. Chem.* **233**: 580. 195

3. Villee, C. A. and D. D. Hagerman: *Endocrinology* **60:** 552. 1957.
4. Villee, C. A.: *Cancer Research* **17:** 507. 1957.
5. Dodds, E. C., L. Goldberg, W. Lawson and R. Robinson: *Nature* **141:** 248. 1938.
6. Hagerman, D. D. and C. A. Villee: *J. Biol. Chem.* **229:** 589. 1957.
7. Villee, C. A. and D. D. Hagerman: *J. Biol. Chem.* **233:** 42. 1958.
8. Hagerman, D. D. and C. A. Villee, unpublished observations.
9. Burstein, S. and S. Lieberman: *J. Biol. Chem.* **233:** 331. 1958.
10. Engel, L. L. in Mechanism of action of steroid hormones, C. A. Villee and L. L. Engel, eds., Pergamon Press, London and New York (in press).
11. Langer, L. J. and L. L. Engel: *J. Biol. Chem.* **233:** 583. 1958.
12. Aizawa, Y, and G. C. Mueller: *Fed. Proc.* **19:** 170. 1960.
13. Barany, E., P. Morsing, W. Mueller, G. Stallberg and E. Stenhagen: *Acta Soc. Med. Upsala* **60:** 68. 1955.
14. Villee, C. A., D. D. Hagerman and P. A. Joel: *Rec. Prog. Hormone Res.* **16:** 49. 1960.

BIOASSAY OF LUTEINIZING HORMONE[1]

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ABSTRACT

The increase in content of radio-iodinated serum albumin of immature rat ovaries is employed as an index of the hyperemia produced by pituitary luteinizing hormone. The assay method consists of the intravenous injection of an LH preparation followed by two micro-curies of radio-iodinated serum albumin two hours later. After 15 minutes, both ovaries are excised and measured for content of the tracer. The regression equation is $Y = 1900 \log X + 2300$ and has an index of precision of 0.14. None of the pituitary hormones except FSH interferes with the specificity of the ovarian response to LH. FSH, when present in large amounts, decreases the slope of the LH dose-response lines to $\frac{1}{4}$ of that given by highly purified LH and thereby invalidates LH potency estimates. After treatment of purified FSH with 6M urea for 24 hours at 40° C, the FSH potency is not affected but 75% of the contaminating LH is inactivated. Purified LH is inactivated to the extent of 98% under the same conditions.

OVARIAN hyperemia has been employed as an end-point for the detection of pituitary luteinizing hormone (LH) and of chorionic gonadotrophin (1–11). In the immature rat or mouse ovary hyperemia occurs several hours after the intravenous injection of these gonadotrophins. It would appear that a rapid and quantitative assay for luteinizing hormone should be feasible by employing a relatively non-diffusable tracer such as radio-iodinated serum albumin for estimating the hyperemic response. The purpose of this report is 1) to describe a technique for the quantitative measurement of ovarian hyperemia by means of radio-iodinated serum albumin, 2) to demonstrate that a linear relationship exists between the dose of LH and the content of radio-iodinated albumin in the ovary, and 3) to describe some observations on the specificity of the reaction to some pituitary hormones.

METHODS

Sprague-Dawley rats, which were employed in these experiments, weighed within 10 grams of each other and were of the same chronological age in each assay. Satisfactory results have been obtained with rats between 24 and 32 days of age. The animals were maintained on Purina laboratory chow. For the purpose of maintaining comparable blood volumes, it is of utmost importance that the animals have access to an adequate supply of water at all times. Animals showing evidence of dehydration should be discarded

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Hormone preparations were dissolved in saline and 0.20 ml. was injected into the tail vein of rats immobilized by wrapping in cheese-cloth. The tails were warmed in a 45° C water bath for 5 to 10 seconds to dilate the veins and facilitate injection.

Labeling of the blood of the animals was accomplished by injecting 2 micro-curies of radio-iodinated serum albumin (RISA) contained in 0.20 ml. of saline. This amount of radio-activity was adequate to produce sufficiently high ratios of ovary to background counts for a counting error of 5% or less. After allowing exactly 15 minutes for mixing of the RISA in the bloodstream, the rats were sacrified with chloroform (approximate time—1 minute). It is essential that the animals are completely expired, since, if they are not, the hyperemia is visibly lessened and the content of RISA in the ovary approaches control values. It is obviously of utmost importance to avoid the loss of blood from the ovary during excision. For this reason the dissection is restricted to a minimum: the ovary with its oviduct, adhering peri-ovarian adipose tissue and capsule, and that portion of the ovarian vein with its branches which lie along the surface of the ovary is removed as a single unit. The amount of adipose tissue which is carried with the ovary is not critical since a volume of this tissue equal to that of the ovary alone does not contain more than 5% of the total radio-activity. Hence, a single cut with dissection scissors is generally sufficient to remove the ovary and surrounding tissue. The exact method of excising the ovary is not critical provided that the same landmarks are used and hemorrhage is avoided. Both undissected ovaries are transferred into a suitable test tube, washed down with one ml. of 2M NaOH and the content of radio-activity measured in a well-type scintillation detector.

Pituitary LH (ovine, NIH-LH-S-1), prolactin (ovine, Armour), and growth hormone (bovine, Armour) were gifts of the Endocrinology Study Section. FSH 264-161-X and LH-227-80 were kindly provided by the Armour Company and were employed as standards each of which was arbitrarily assigned unit potency per milligram. Other pituitary preparations have also been employed where indicated and have been purified according to methods described elsewhere (12). Radioiodinated human serum albumin (RISA) was purchased from the Abbott Laboratories, North Chicago, Illinois. Statistical calculations were performed according to the methods detailed by Burn *et al.* (13).

RESULTS

Development of Hyperemia following LH: Luteinizing hormone produced a marked hyperemic response in the ovaries as early as one hour after intravenous injection. Figure 1 shows the magnitude of the responses obtained at various time intervals following LH injection. In this and other experiments, it has been consistenly observed that the maximal content of RISA in the ovaries occurs between 2 and $2\frac{1}{2}$ hours after LH administration. Hence, in the standard procedure for the assay of LH, 2 hours were allowed for the development of the hyperemia after which time RISA was injected and the animals were sacrificed 15 minutes later.

Relationship Between RISA Content of Ovaries and Dose of LH: The relationship between dose of LH and the hyperemic response as measured by the content of RISA is shown in Figure 2 for two LH preparations. Armour LH 227-80 was used as a standard and LH XIII-159-3 was a partially purified preparation obtained by fractionation with IRC-50 (12). A linear dose response relationship was obtained between 0.5 and 2.0 μg of the standard. The regression equation for the standard was $Y = 1900 \log$

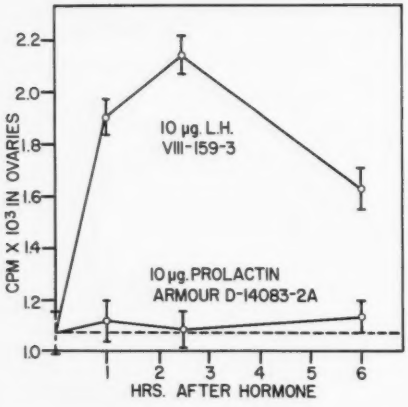

FIG. 1. Rate of development of ovarian hyperemia following injection of partially purified sheep LH. Vertical bars show standard error; five rats per point.

X+2300 and the index of precision, λ, was 0.14. Closely similar results have been obtained with the NIH-LH-S-1 preparation which was equipotent with the Armour standard when assayed by this method. The maximal degree of hyperemia which can be achieved by LH administration amounts to 110–115% over the control value. Occasionally the content of radioiodinated serum albumin may be 200% or higher; such values are false since they can be traced to contamination of the ovaries with extraneous blood during excision.

Specificity of Response: Figure 1 shows that prolactin produces no hyperemia either at 1, 2½ or 6 hours following the injection of 10 μg of the hormone. This is in contrast to the positive effect observed by other investi-

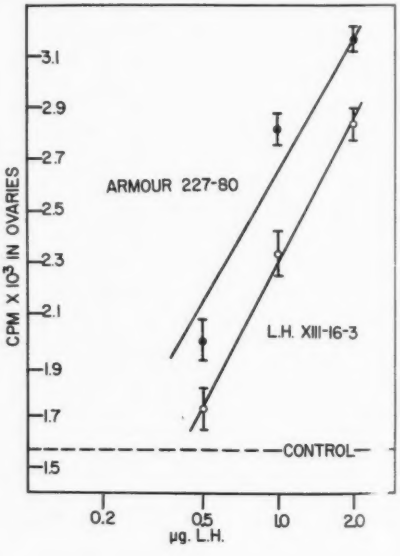

FIG. 2. Dose-response lines for Armour LH 227–80 and a partially purified sheep LH XIII-16-3. The respective regression equations were $Y=1950\ X+2660$ and $Y=1839\ X+2300$. The potency of LH XIII-16-3 was 0.66 with a range of 0.58 to 0.75 at 95% confidence limits. The mean index of precision was 0.12.

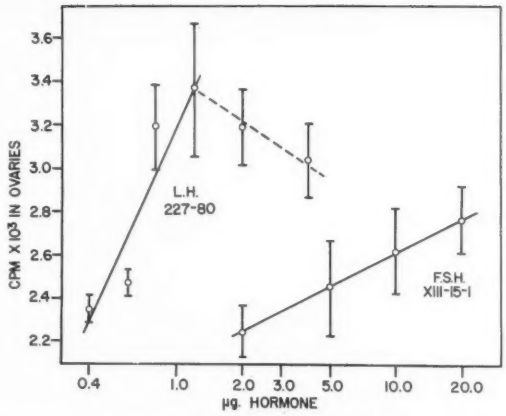

FIG. 3. Dose-response line for Armour LH 227-90 showing plateauing of responses at high doses of LH. Dose-response line of purified sheep FSH prepared by zone electrophoresis (12). Vertical bars show standard error; four rats per point.

gators (8, 11). A likely explanation of the discrepancy may lie in the degree of purity of the different prolactin preparations employed. The prolactin employed in these studies, Armour Lot No. R-10109, was shown to be free of LH and FSH at a total dose of 10 mg. as judged by the ventral prostate method (14) and the HCG augmentation test (15). Furthermore, the injection of graded doses of LH as in Figure 2 but containing a constant level of prolactin (10 μg) gave a regression equation which was not statistically different at 95% confidence limits from that obtained with LH alone. Similarly, 25 or 50 U.S.P. milli-units of corticotropin (sheep) were without effect when injected either singly or with graded doses of LH. Pitressin (Parke, Davis) in doses of 0.01 or 0.1 I.U. and growth hormone in 100 microgram doses were without effect when injected either singly or in combination with graded doses of LH.

All preparations of TSH so far tested have shown the presence of 1.8 to 20% LH relative to the Armour LH 227-60. Since the LH content of TSH decreased from 20% to 1.8% as the potency of the TSH increased from 1 to 40 U.S.P. units per mg., it is unlikely that TSH has any significant hyperemic effect on the ovary. Moreover, the simultaneous administration of purified TSH (10 U.S.P. units per mg.) with graded doses of LH showed no augmentation of the LH response nor change in slope of the dose-response line.

All preparations of FSH obtained in the course of FSH purification from sheep pituitaries (12) produced ovarian hyperemia, when administered in high doses. Figure 3 shows the dose-response line obtained with a purified sheep FSH which had a potency 25 times Armour FSH No. 264-141X. The equation of the dose-response line was $Y = 512 \log X + 2100$ showing that

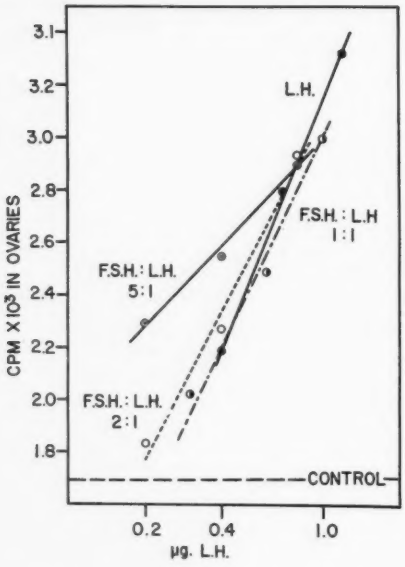

FIG. 4. Effect of different ratios of FSH to LH on the dose-response line of purified LH (NIH-LH-S-1). FSH (potency 50 times Armour FSH 264-161X) and LH (NIH-LH-S-1) combined in the indicated ratios and injected as single solution. Only FSH:LH in the 5:1 ratio had a significantly lower slope at $P = 0.05$.

the slope is only about $\frac{1}{4}$ that given by purified LH. In crude extracts or fractions, where the FSH to LH ratio was nearly the same as that found in unfractionated pituitary extracts, the slopes of the dose-response lines were equal to that of highly purified LH (Armour 227-80). However, after removal of the major part of the LH and inert impurities so that the FSH potency ranged from 2 to 60 times standard (Armour 264-161X or Li [16]), the slope of the dose response lines ($Y = 550 \log X + 1590$) again was only $\frac{1}{4}$ that given by highly purified LH. These results suggested that the ratio of FSH to LH influenced the slope of the dose response lines. To examine this possibility, three-point assays were performed on solutions in which FSH and LH were deliberately combined in various ratios. As shown in Figure 4, FSH:LH ratios of 1:1 and 2:1 yielded dose-response slopes which were not significantly different from those given by purified LH alone. When the ratio of FSH:LH was 5:1, the slope of the dose-response line ($Y = 1180 \log X + 3087$) was significantly lower at $P = 0.05$ from that given by LH alone or the 1:1 and 2:1 FSH:LH solutions. Therefore, it can be concluded that potency of LH cannot be estimated validly by ovarian hyperemia when FSH is present in large amounts. Fortunately, this situation can be readily detected by the non-parallelism of slope between heavily contaminated LH and a relatively FSH-free LH standard such as Armour 227-80 and NIH-LH-S-1, for example. The diminution in slope of the dose-response lines caused by high FSH:LH ratios such as those present in purified FSH or in synthetic mixtures clearly indicates that FSH modifies the responsiveness of the ovary to the production of hyperemia by LH. Mo-

TABLE 1. STABILITY OF LH AND FSH IN 6M UREA

Preparation	Treatment[1]	Mean LH potency (% standard)[2]	Mean FSH potency (× standard)[3]
FSH	1. untreated	8	10
	2. 6 hrs., 25°	1	9
	3. 12 hrs., 25°	3	10
	4. 24 hrs., 25°	2	11
	5. 24 hrs., 40°	2	10
LH (NIH-LH-S-1)	6. untreated	100	—
	7. 24 hrs., 25°	20	—
	8. 9 hrs., 40°	4	—
	9. 24 hrs., 40°	2	—

[1] One per cent solutions of either FSH or LH incubated for the indicated times in 6M urea—0.025M Na_2HPO_4 − 0.025M NaH_2PO_4, pH 7.0. For assay solutions diluted with saline to appropriate hormone concentrations; maximum concentration of urea which was injected with the hormone did not exceed 0.2 ml. of 0.16M urea. Potency estimates relative to standards were derived from 6 point design containing four animals per point. Levels of significance: FSH, within 2 to 5, $P > .05$; between 1 and 2 to 5, $P < .05$; LH, between 8 and 9, $P > .05$; between 6 and 7, $P < .05$; between 7 and 8 or 9, $P > .05$.

[2] Armour LH No. 227–80.

[3] Armour FSH No. 272-161X.

over, the presence of large amounts of FSH augments (10) the hyperemic response of the ovary to LH as judged by the increased hyperemia at the lower doses of LH in the 5:1 FSH:LH dose-response line shown in Figure 4.

The question as to whether FSH itself possesses a hyperemic action independent of contaminating LH has been difficult to ascertain, since all preparations of FSH studied so far contained varying amounts of LH. With the hope of selectively inactivating LH, 6M urea buffered at pH 7.0 was tested for its effect on the potency of FSH and of LH. As can be seen from Table 1, incubation of purified FSH in this medium produced no reduction in potency whereas purified LH lost 98% of its original potency after 9 or more hours at 40°. With regard to LH contamination in FSH, 75% of the original LH contamination was lost under the same conditions of treatment. It should be noted that the estimation of LH contamination in the untreated FSH was obtained by the ventral prostate method (14) since lack of parallelism between FSH and the LH standard discussed above, prohibited a valid estimate of potency by the hyperemia method. After treatment with 6M urea, the slopes of the dose-response lines were no longer significantly different from those given by the LH standards so that estimates of LH content were derived from hyperemia data. The two important features of these results are: 1) urea treatment increased the slopes of the FSH dose-response lines to that given by LH and, 2) urea did not inactivate as much apparent LH contamination in FSH as in the case of purified LH; i.e., 75% as compared to 98%. The identity of slopes between the urea treated FSH and the LH standard would tend to support the hypothesis that at appropriate ratios of FSH:LH, the FSH causes an increased sensitivity to the lower doses of LH, thereby reducing the slope of

the LH dose-response line. The persistence of as much LH contamination in the urea treated FSH as noted above is difficult to explain. It may be that FSH tends to stabilize traces of LH from inactivation by urea or that FSH has some intrinsic hyperemia-producing activity. However, no data are available to support either of these possibilities.

COMMENT

The assay method described here has been employed in estimating the potency of various pituitary fractions obtained in the course of LH purification (12) and in the preparation of NIH-LH-S-1 for the Endocrinology Study Section. The chief disadvantage of the method is the impossibility of estimating LH potency in the presence of large amounts of FSH. For routine assay of LH potency of fractions such as those obtained in LH purification and stability studies, the method is extremely useful because of its speed and precision. Thus, it is possible to perform three-point assays on three unknown preparations against a standard employing four animals per group and obtain the results at the end of the same day. Two operators and injections at one-minute intervals are required for a schedule of this kind. The injections and autopsies can be performed in the forenoon and the ovary samples measured in the afternoon.

Acknowledgment

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REFERENCES

1. Eberson, F. and M. H. Silverberg: *J. Am. Med. Assoc.* **96:** 2176. 1931.
2. Salmon, U. J., S. G. Geist, A. A. Salmon and I. L. Frank: *J. Clin. Endocrinol.* **2:** 167. 1942.
3. Zondek, B., F. Salmon and R. Black: *J. Am. Med. Assoc.* **128:** 939. 1945.
4. Zondek, B. and F. Salmon: *J. Clin. Endocrinol.* **7:** 159. 1947.
5. Kaminester, S.: *Amer. J. Obst. Gyn.* **47:** 265. 1944.
6. Sturgis, S. H. and V. C. Politou: *Endocrinology* **48:** 435. 1951.
7. Lloyd, C. L.: *J. Clin. Endocrinol.* **9:** 636. 1949.
8. Albert, A. and J. Berkson: *J. Clin. Endocrinol.* **11:** 805. 1951.
9. Farris, E. J.: *Am. J. Obst. Gyn.* **52:** 14. 1946.
10. Kupperman, H. S., W. H. McShan and R. K. Meyer: *Endocrinology* **43:** 275. 1948.
11. Payne, R. W.: *Proc. Soc. Exper. Biol. and Med.* **77:** 242. 1951.
12. Ellis, S.: *J. Biol. Chem.* **233:** 63. 1958.
13. Burn, J. H., J. D. Finney and L. G. Goodwin: *Biological Standardization*, 2nd edition, Oxford University Press, London, 1950.
14. Greep, R. O., H. B. Van Dyke and B. F. Chow: *Endocrinology* **30:** 635. 1942
15. Steelman, S. L. and F. M. Pohley: *Endocrinology* **53:** 604. 1953.
16. Li, C. H., M. E. Simpson and H. M. Evans: *Science* **109:** 445. 1949.

A STUDY OF THE VIRILIZING EFFECT OF PROGESTOGENS ON THE FEMALE RAT FETUS

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ABSTRACT

A series of progestogenic steroids has been evaluated for: (1) virilizing activity during gestation on the female fetus of the rat, (2) androgenic activity on the spayed rat seminal-vesicle weight and on the chick-comb, and (3) progestational activity on the rabbit uterus. The results indicate that: (1) the 17α-hydroxyprogesterone-derivatives, even in extremely high dosage, exhibit no masculinizing properties; (2) the alkylation of 17α-hydroxyprogesterone-derivatives may lead to virilizing activity; (3) 17α-ethinyl-testosterone has less virilizing activity than any of its derivatives that were tested; and (4) the most virilizing steroid among the 17α-alkyl-19-nor-testosterone derivatives is the 17α-methyl-compound; the 17α-ethinyl-compound and its 17β-acetate are remarkably less virilizing; and the 17-alkyl-estrenole has the least virilizing activity of this group. Preliminary animal studies to determine the relative potency of these activites should be undertaken with all compounds that are proposed for use as therapeutic agents in human pregnancy disorders.

THE development of synthetic progestogens has resulted in the preparation of a number of compounds which are superior in progestogenic potency to the relatively weak progesterone. Some of these new substances have been derived from 17α-hydroxyprogesterone (13), and some are derivatives of 17α-alkyl-19-nor-testosterone (2, 20, 10, 4, 21, 7, 17, 18). The last group has the advantage of marked oral activity. Because these compounds are similar to 17α-ethinyl-testosterone (11) and related to testosterone, the possibility that these agents also may have androgenic properties has become a problem of importance. Therefore, several investigators have studied the androgenic activity of various progestogens by measuring the increase of weight of the seminal vesicles and ventral prostate in castrated male rats (23, 15, 24, 25).

A number of cases have been reported of human female pseudohermaphroditism following the administration of progestogens during gestation (30, 19, 14, 8, 29). Some of these cases resulted from the administration of 17α-ethinyl-testosterone (30), and some from the use of 17α-ethinyl-19-nor-testosterone (19, 14, 8, 20). The changes induced in the genital tract appear to depend upon the duration of the gestation when the treatment is started and dosage of the agent that is given. When the administration

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was started in the 13th week of gestation, a fusion of the labioscrotal folds was observed; when therapy was initiated later in the pregnancy, an enlargement of the clitoris took place. When diethylstilbestrol or estrone sulfate was employed simultaneously with the progestational medication, the masculinizing effects seemed to be enhanced. The virilizing action of these compounds upon the female fetus seems to be attributable to the androgenic properties of the steroids, since androgens administered during pregnancy are known to cause masculinization in human beings and in animals (9, 6, 12, 16, 1, 5, 22, 26, 27, 28). In view of these observations, we have undertaken a series of experiments to determine the possible virilizing properties of various progestogens on the female fetus of the rat.

METHODS

Vaginal smears were taken from mated females and studied for sperm. Animals with positive smears were separated, and treatment was begun on the 16th and ended on the 19th day of the pregnancy. All compounds were administered by the subcutaneous route. On the 20th day, the fetuses were removed from the uterus by Caesarean section. The anogenital distance was measured and the fetuses separated into males and females. Cross-sections of the pelvic region of the females were prepared and studied under the steromicroscope for the development of the vagina. Later, corresponding sections were studied microscopically. These results will be published elsewhere. A total of 13,600 female fetuses have been examined. As standards, testosterone-17β-propionate was used as an androgen and progesterone as a progestogen. The androgenic activity was assayed by the Dorfman's chick-comb inunction method (3). The progestogenic activity of all compounds was determined by a modified Clauberg test.

RESULTS

The first group of compounds to be discussed are the 17α-hydroxy-progesterone-derivatives (Figs. 1 and 2). These substances differed significantly in their progestational activity. The most active compound was the 19-nor-17α-hydroxy-progesterone-acetate, which was found to be 10 times as potent than the corresponding 19-CH_3 compound. The 19-nor-17α-hydroxy-progesterone-caproate proved to be two orders of 10 more potent than the 19-CH_3 steroid. With these two hydroxyprogesterone esters, no increase of the seminal-vesicle weight was observed in the dosages employed; the curves of the chick-comb body-weight ratio ran parallel to the baseline; the anogenital distance remained normal; and no inhibition of the vaginal development was detected.

The methylation of C_6 in 17α-hydroxy-progesterone-acetate (Figs. 3 and 4) completely changed the effect of the original compound. Whereas the progestogenic potency was not increased by this structural change, the C_6 methylation induced a slight increase of the seminal-vesicle weight which became evident at the higher doses and a slight regression in the chick-comb body-weight ratio. Both of these androgenic manifestations occurred at doses which both exceeded the progestogenic range. The virilizing effect of the C_6 methylated 17α-hydroxy-progesterone acetate upon

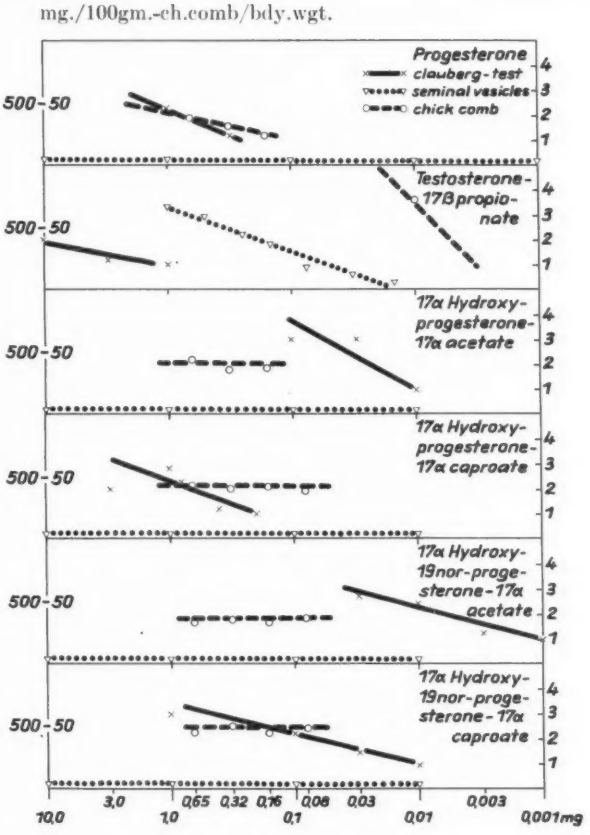

FIG. 1

the female fetuses was striking, and almost as marked as in the testosterone-17β-propionate treated group.

The group of 17α-alkyl-testosterone-derivatives (Figs. 3 and 4) showed a slight increase of progestogenic potency with the additional alkylation in C_6 and C_{21}. However, the chick-comb test gave a more pronounced regression line with 17α-ethinyl-testosterone. The influence of the compounds in this group upon the increase of the anogenital distance, and the effect upon the inhibition of vaginal development was the same; and the masculinizing activity was found to be present in doses within the progestogenic range.

The last compounds to be considered are the 19-nor-17α-alkyl-testosterone-derivatives (Figs. 5 and 6), a group which is important because of its oral activity. The first compound, 17α-methyl-19-nor-testosterone, is a steroid with considerable progestogenic potency, but also with marked

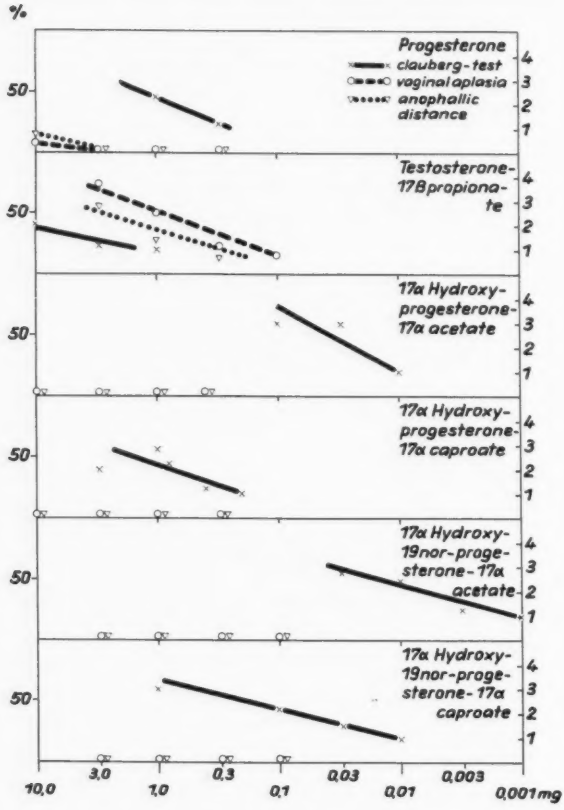

FIG. 2

androgenic properties, as can be seen by the seminal-vesicle weight increase and the testosterone-17β-propionate-like regression line in the chick-comb test. The influence upon the anogenital distance and the vaginal development of this steroid was equal to that of testosterone-17β propionate and occurred with doses within the progestogenic range. 17α-ethinyl-19-nor-testosterone and its 17β-acetate showed a difference in progestogenic potency; the 17β-acetate was 5 times as potent. With both substances, the androgenic effect upon the seminal vesicles became evident only in high doses, and the chick-comb test line showed a moderate regression. There were no significant differences in the increase of the anogenital distance and the rate of vaginal aplasia that were induced by these compounds. In the latter, the masculinizing effect seemed to occur with doses that were a little further from the progestogenic range.

The significantly less potent progestogens, 17α-ethinyl-$\Delta^{5(10)}$-androstene-17β-ol-3-one (Figs. 5 and 6), had a slight androgenic effect upon the sem

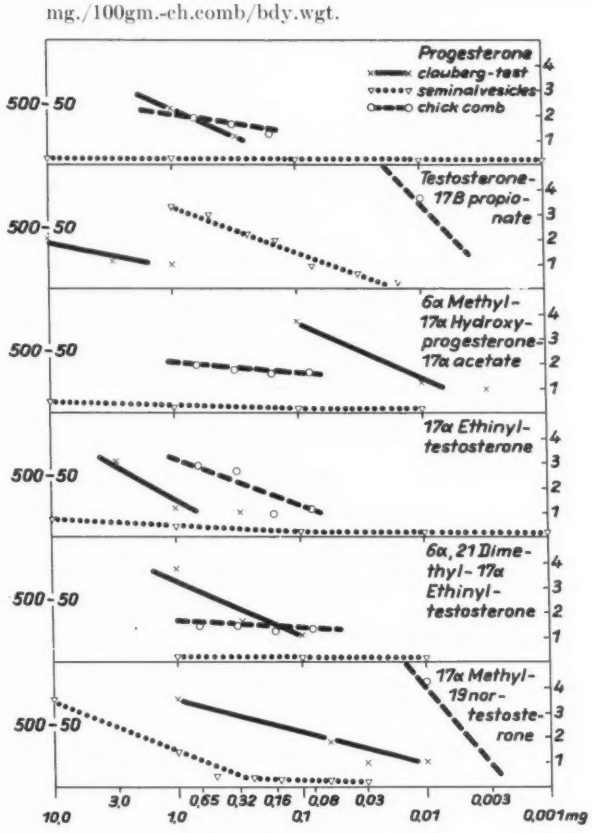

FIG. 3

inal vesicles, and a regression of the chick-comb body-ratio line which was similar to that of the 17α-ethinyl-19-nor-testosterone. However, the virilizing effect of the $\Delta^{5(10)}$ compound on the female fetuses was much more pronounced, and occurred with doses within its progestogenic range.

The final compound, 17α-allyl-estrenole (Figs. 5 and 6), was a rather weak progestogen with a striking discrepancy between the seminal-vesicle weight increase and the chick-comb body-weight ratio: whereas the seminal vesicles showed no increase in weight, the chick-comb test had a marked and steep regression line. The masculinizing effect of this compound upon the female fetuses first became evident as an increase in the anogenital distance, but with higher doses there was a significant inhibition of the vaginal development.

An attempt was made with some of the compounds to study the influence of treatment administered at different stages of pregnancy or throughout

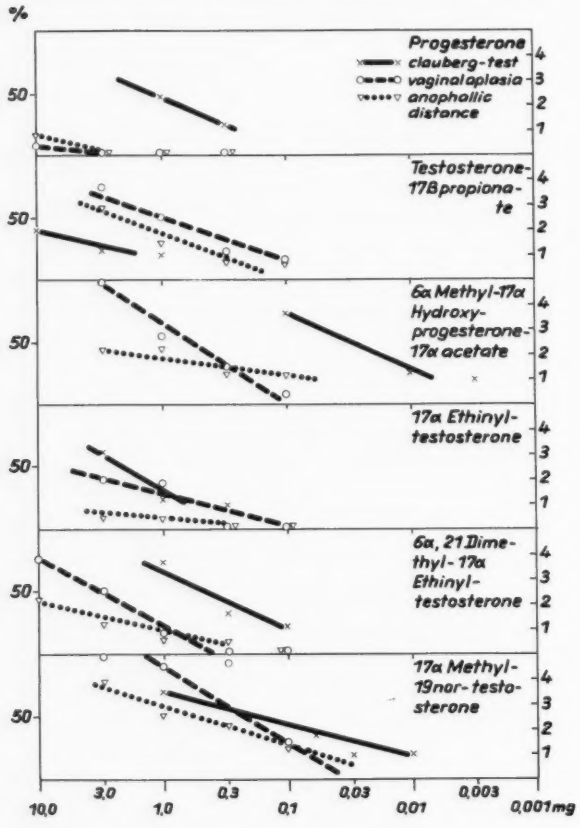

FIG. 4

the whole period of gestation. The results are given in Figure 7. Only 17α-ethinyl-testosterone inhibited the vaginal development when treatment was begun in the first trimester and ended on day 8; the same result was obtained with treatment started in the second trimester of gestation. 17α-methyl-19-nor-testosterone was found to be virilizing when the medication was begun in the second or in the third trimester.

DISCUSSION

These studies indicate that the virilizing properties of a steroid are not necessarily paralleled by the androgenic activity of the compound as determined by the increase in the weight of the seminal vesicles in the spayed male rat or by the increase in the chick-comb body-weight ratio. This discrepancy was most marked in the compound 6α-methyl-17α-hydroxy-progesterone-acetate, which in our investigations was as virilizing as

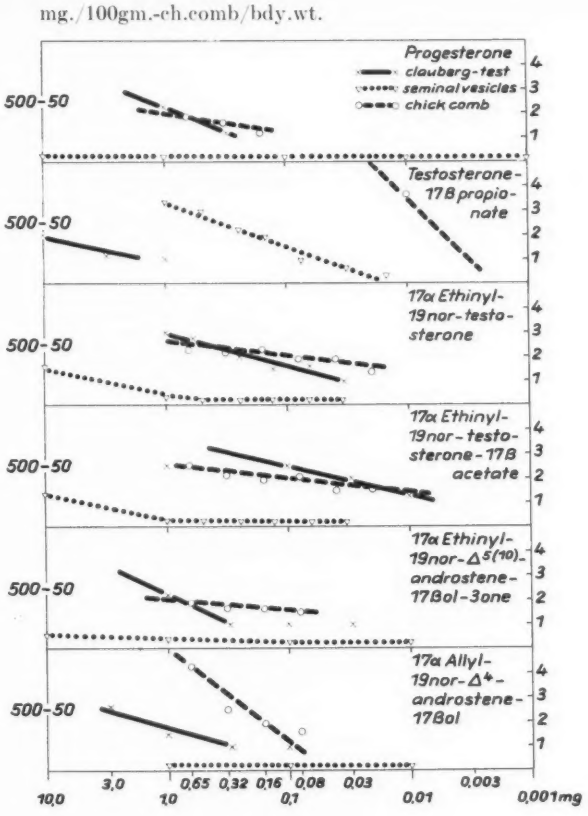

FIG. 5

testosterone-17β-propionate. We believe that a difference in metabolism must be responsible for this undesirable activity. The 3-keto-Δ^4-system seems to be protected from this action by an additional alkylation in ring A or B.

In the case of 17α-allyl-Δ^4-estrene-17β-ol, in contrast to all other steroids, the 3-OH group (usually formed in metabolism by reduction in the A ring) is missing. This group conjugates with glucuronic acid thus facilitating rapid excretion, and an absence of this group apparently explains the prolonged action of this steroid. In unpublished experiments (27), we have found that methylation apparently is much more effective in protecting the A ring than ethylation.

The results herein reported emphasize the necessity of performing preliminary studies in animals with all compounds which are being considered as agents for the treatment of habitual and threatened abortion in human

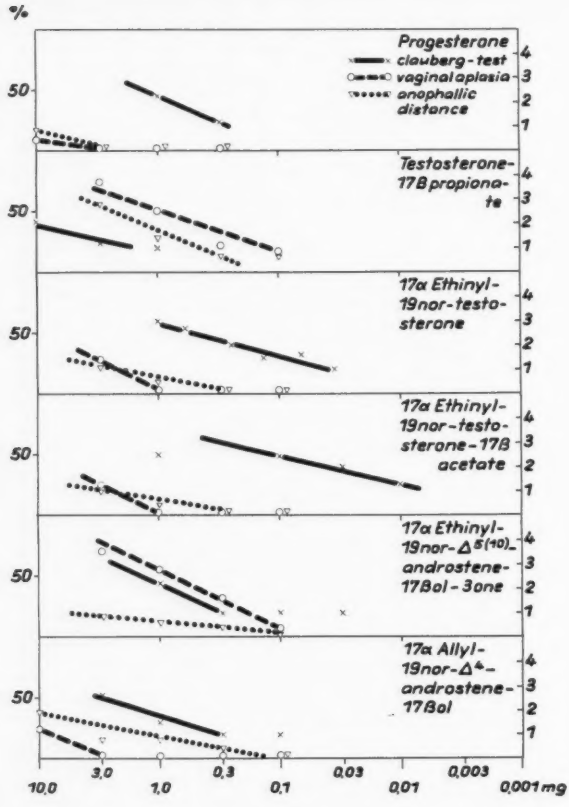

FIG. 6

ED_{50}	5-8 d	10-13 d	16-19 d	5-20 d
Testosterone-17β propionate	> 1	> 1	1	1
Progesterone	> 1	> 1	100	100
17α Hydroxyprogesterone-17α acetate	—	—	> 100	—
17α Hydroxyprogesterone-17α caproate	> 100	> 100	> 200	> 100
17α Hydroxy-19nor-progesterone-17α acetate	—	—	> 30	—
17α Hydroxy-19nor-progesterone-17α caproate	—	—	> 30	—
6α Methyl-17α hydroxyprogesterone-17α acetate			1	1
17α Ethinyl-testosterone	< 1	< 1	10	1
6α, 21 Dimethyl-17α ethinyl-testosterone	—	—	10	—
17α Methyl-19nor-testosterone	> 1	< 1	1	1
17α Ethinyl-19nor-testosterone	> 1	> 1	30	3
17α Ethinyl-19nor-testosterone-17α acetate	> 1	> 1	30	3
17α Ethinyl-19nor-Δ5(10)-androstene-17βol-3one	—	—	10	—
17α Allyl-19nor-Δ4-androstene-17βol	—	—	100	—

FIG. 7

beings. The evaluation of the virilizing activity of such substances can be carried out easily and reliably on the fetus of the female rat.

REFERENCES

1. Bur, G. E., E. Montouri and A. Korompay: *Bol. Soc. Obstet. y Ginec.* (Buenos Aires) **XXXVIII:** 536. 1959.
2. Djerassi, C., L. Miramontes and G. Rosenkranz: Abstr. Meet. Div. Med., Am. Chem. Soc. Milwaukee, Wis. **18:** 1952.
3. Dorfman, R. I.: *Endocrinology* **42:** 1. 1948.
4. Ferin, J.: *Ann. Endocrinol.* **17:** 261. 1956.
5. Forsberg, J.-G.: *Acta Endocrinol.* **33:** 520. 1960.
6. Green, R. R., M. W. Burrill, and A. C. Ivy: *Am. J. Anat.* **65:** 415. 1939.
7. Greenblatt, R. B.: *J. Clin. Endocrinol. & Metab.* **16:** 869. 1956.
8. Grumbach, M. M., J. R. Ducharme and R. Moloshok: *J. Clin. Endocrinol. & Metab.* **19:** 1369. 1959.
9. Hamilton, J. B. and J. Wolfe: *Anatom. Rec.* **70:** 433. 1937/38.
10. Herz, R., W. W. Tullner and E. Raffelt: *Endocrinology* **54:** 2. 1954.
11. Inhoffen, H. H. and W. Hohlweg: *Naturwissenschaften* **26:** 96. 1938.
12. Jost, A.: *Geburtsh. u. Frauenhk.* **14:** 687. 1954.
13. Junkmann, K.: *Arch. Exp. Path. & Pharm.* **223:** 244. 1954.
14. Magnus, E. M.: *Tidssk. Norske Laegefor.* **80:** 92. 1960.
15. McGinty, D. A. and C. Djerassi: *Ann. N. J. Acad. Sc.* **71:** 500. 1958.
16. Mey, R. and H. Scheid: *Geburtsh. u. Frauenhk.* **19:** 783. 1959.
17. Miyake, T. and G. Pincus: *Endocrinology* **63:** 816. 1958.
18. Moggian, G.: *Endocrinology* **64:** 363. 1959.
19. Napp, J.-H.: 6. Symp. Dtsch. Ges. Endokrin. Kiel, 1959.
20. Overbeek, G. A. and J. deVisser: *Acta Endocrinol.* **22:** 318. 1956.
21. Pincus, G., M. C. Chang, E. S. E. Hafez and M. X. Zarrow: *Science* **124:** 890. 1956.
22. Revesz, C., C. I. Chappel and R. Gaudry: *Endocrinology* **66:** 140. 1960.
23. Saunders, F. J. and V. A. Drill: *Endocrinology* **58:** 567. 1956.
24. Schneider, W. and H. Rauscher: *Arch. int. pharmacodyn.* **CXIX:** 345. 1959.
25. Suchowsky, G. K.: unpublished data, 1958.
26. Suchowsky, G. K. and K. Junkmann: *Ztschrft. f. Frauenheilkd. und Geburtshf.*, 1960 (in press).
27. Suchowsky, G. K.: unpublished data, 1960.
28. Suchowsky, G. K. and K. Junkmann: 1st International Congress of Endocrinology, Copenhagen, July, 1960.
29. Wilkins, L.: *J.A.M.A.* **172:** 1026. 1960.
30. Wilkins, L., H. W. Jones, G. H. Holman and R. S. Stempfel: *J. Clin. Endocrinol. & Metab.* **18:** 559. 1958.

NOTES AND COMMENTS

PITUITARY GONADOTROPHINS DURING THE OVULATORY CYCLE OF THE DOMESTIC FOWL

ABSTRACT

Mature laying hens were killed 8 hours before or 2 hours after ovulation. The pituitary glands were collected at these times, pooled, and their gonadotrophic potency compared. Three bioassay methods were used in making the comparison—the seminal vesicle and uterine weight tests in immature mice and the ovulation test in pregnant mice.

The gonadotrophic potency of pituitaries tended to be higher in birds killed before ovulation than in those killed after ovulation; only in one experiment, however, were the results statistically significant.

The results obtained are compatible with the view that before ovulation a gonadotrophin is released from the pituitary of fowls, but because of the relative non-specificity of the bioassay methods used, it was not possible to determine whether this gonadotrophin was FSH or LH.

According to Fraps and his associates (1) ovulation is caused by a substance termed "ovulation inducing hormone" (OIH), which is believed to be identical with LH. These workers have also shown (2, 3) that the anterior pituitary is essential for this process and that ovulation is associated with the liberation of the hormone or hormones from the gland. The main purpose of the present study was to obtain more accurate information on this point by comparing the gonadotrophin concentration in the pituitaries of mature laying hens before ovulation with that after ovulation.

MATERIALS AND METHODS

Three experiments in all were conducted. The birds to be used were housed in single cages in a battery house at 20 weeks of age and they were kept for 14 hours in the light and for 10 hours in the dark. Food and water were available ad libitum.

Timing of ovulation

Times of oviposition were noted over a period of two weeks. From this it was possible to estimate "lag," i.e. the time in excess of 24 hours between successive oviposition in a clutch (4). When times of oviposition could be predicted with some accuracy it was possible to determine the approximate time of ovulation, which normally follows oviposition, by adding 45 minutes to the observed time of lay (5). In birds killed at times judged to be 8 hours before ovulation was due to take place, a fully shelled egg could be palpated in the uterus. In birds killed approximately 2 hours after ovulation, it was possible, by digital palpation through the rectum, to check that the liberated ovum was present in the magnum.

In experiments 1 and 2 the mean lag period was calculated jointly for all birds, but in experiment 3 it was determined for individual birds.

Measurements taken at sacrifice and method of removing the pituitary

Immediately after sacrifice, the glands were removed using the technique described by Hill and Parkes (6). They were crushed between two large microscope slides and were dried in a warm current of air, according to the method of Kupperman and his associates (7). The glands were subsequently scraped off the slides, using a single edged razor blade, were weighed, ground into powder and stored in a vacuum desiccator containing anhydrous calcium chloride. After removal of the pituitaries, the oviduct and ovaries were carefully removed the organs were freed from fat and connective tissue and were weighed on a torsion balance.

The organ weights, together with data on egg production, were used to check that the two groups of birds in each experiment were comparable.

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Bioassay methods

Assays on the unfractionated pituitary powder were performed by three methods.

(i) Test depending upon the enlargement of the seminal vesicle in intact, immature mice (8, 9)—experiments 1 and 3. Five animals per dosage group were used and were injected intraperitoneally twice daily for 4 days before necropsy on the 5th day.

(ii) Test depending upon the enlargement of the uterus in intact, immature mice (10)—experiment 3. Five animals per dosage group were used and were injected subcutaneously twice daily for 4 days before necropsy on the fifth day.

(iii) Test depending upon ovulation in pregnant mice (9, 11)—experiment 2. Ten animals per dosage group were used and were injected once intraperitoneally; after 18 hours the Fallopian tubes were examined for the presence of ova.

All doses of pituitary powder were calculated in "Pituitary Equivalents" (P.E.). One P.E. was defined as the amount of pituitary powder equal to the mean dried weight of the glands contained in each pooled sample of powder. The powder was prepared for injection by suspension in distilled water, requisite dose levels being obtained by serial dilution. A total volume of 2 cc. was injected in tests (i) and (ii) and 0.5 cc. in test (iii).

Statistical methods

In the seminal vesicle and uterine weight tests, the organ weights were transformed to Log_{10} organ weight-body weight ratios. This transformation has been shown to reduce variation due to differences in body weights of the assay animals and to control the tendency of the variance about the mean to increase at higher dose levels (12, 13).

The ovulation test in mice depends on a quantal (all-or-none) response, and, because of this, the figures obtained were transformed into probits. The methods used in conducting the probit analysis and the analysis of variance, in performing validity tests and in calculating the indices of precision, relative potencies and limits of error, have been described elsewhere (14, 15). It should be emphasised that in all assays listed in Table 1, validity tests were satisfactory and that, in particular, there was no significant deviation from parallelism in any assay.

RESULTS

The gonadotrophic potencies obtained with the 3 assay methods, fiducial limits of error and the precision of the assays are listed in Table 1.

The gonadotrophic potency of pituitaries collected before ovulation tend, on average, to be higher than that in glands collected afterwards. However, only in the case of experiment 3, where both the seminal vesicle weight and uterine weight methods were used, was the figure for "t" in the range generally accepted as being significant.

The index of discrimination (15) has been widely used as a means of detecting the presence or absence of qualitative differences (16). The index is calculated by dividing the mean potency ratio in one test by the corresponding figure in another. It is close to unity when 2 substances possessing identical biological activity are assayed; it diverges considerably from unity when the materials being assayed are qualitatively different. In experiment 3 the index of discrimination (seminal vesicle/uterus) was 1.81; this figure suggests that, with this combination of assays, the presence of a qualitative difference was not detected.

DISCUSSION

In this study, the times of sacrifice of the birds—8 hours before ovulation and 2 hours after ovulation—were selected in the hope that maximal differences of pituitary gonadotrophic potency might be obtained. It appeared reasonable to assume that, in birds killed 8 hours before ovulation, the stimulus for the release of the gonadotrophin causing ovulation, might not have been received, and that the pituitaries of such birds might be expected to contain maximum amounts of gonadotrophins (2). On the other hand, in birds killed 2 hours after ovulation, "ovulating hormone" would presumably have been released and, under these circumstances, the pituitary gonadotrophic potency might be expected to be low. It has been suggested (17) that the interval between stimulation of the pituitary and ovulation itself, may vary from 8–14 hours; if this should be true, then the glands collected 8 hours before ovulation might not contain maximal amounts of gonadotrophin, and the times selected for sacrifice might not have been optimal.

The present study has not yielded positive information as to whether pituitary FSH

TABLE 1. ESTIMATES OF RELATIVE POTENCY, PRECISION AND FIDUCIAL LIMITS OF ERROR WITH 3 BIOASSAY METHODS

Experiment	Method of bioassay	No. of animals	Dose (P.E.)	Responses from glands collected "before" ovulation		Responses from glands collected "after" ovulation		Relative† potency	Fiducial limits of error	Index of Precision	Significance
				Mean $\log_{10}$ organ wt. body wt. ratio	Standard error ±	Mean $\log_{10}$ organ wt. body wt. ratio	Standard error ±				
1	Seminal Vesicle Weight (Mice)	10 5 5 5	0 $\frac{3}{4}$ $1\frac{1}{2}$ 3	0.98889 1.07453 1.34904 1.47129	0.06197 0.11256 0.14913 0.12017	— 1.09726 1.23096 1.45240	— 0.07014 0.05030 0.15206	0.84	1.15–0.60	0.184	N.S.*
3	Seminal Vesicle Weight (Mice)	15 5 5 5	0 $\frac{3}{4}$ $1\frac{1}{2}$ 3	0.93730 1.04711 1.18316 1.28889	0.14923 0.13899 0.11305 0.08444	— 0.93444 0.97870 1.22722	— 0.18696 0.06140 0.10784	0.37	0.67–0.30	0.274	P = 0.01
	Uterine Weight (Mice)	10 5 5 5	0 $\frac{3}{4}$ $1\frac{1}{2}$ 3	1.85655 2.06900 2.17278 2.45023	0.05797 0.10173 0.07169 0.16099	— 2.02272 2.16721 2.23952	— 0.02236 0.08385 0.13798	0.67	0.84–0.44	0.214	P = 0.05
				No. of animals ovulating	% Ovulation	No. of animals ovulating	% Ovulation				
2	Ovulation Test (Pregnant Mice)	10 10 10 10 10	$\frac{3}{16}$ $\frac{3}{8}$ $\frac{3}{4}$ $1\frac{1}{2}$ 3	2 3 6 9 9	20 30 60 90 90	1 2 7 8 8	10 20 70 80 80	0.77	1.50–0.38	0.497‡	N.S.*

* "N.S." = not significant
† = No. of P.E. of the pituitaries collected before ovulation which are equal in potency to 1 P.E. of the pituitaries collected after ovulation.
‡ = Calculated from 1/b.

or LH is responsible for ovulation in the hen. This is largely due to the fact that none of the assay methods used was specific for one or other hormone. The finding of an index of discrimination in experiment 3 of less than 2.00 also suggests that there was no qualitative difference in the gonadotrophin content of the two groups of glands. It would be of considerable interest to repeat this work, using assay methods such as the ovarian "augmentation" test in mice (18), and the hypophysectomised rat prostate test (19, 20) which are generally assumed to be reasonably specific for FSH and LH activities respectively.

The evidence presented herein is consistent with the view that release of a hormone (or hormones) from the pituitary subsequently brings about ovulation. To obtain more precise information it would be necessary to measure pituitary gonadotrophin potencies at other selected time intervals using bioassay methods specific for LH and FSH.

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REFERENCES

1. FRAPS, R. M., M. W. OLSEN AND B. H. NEHER: *Proc. Soc. exp. Biol. Med.* **50:** 308. 1942.
2. ROTHCHILD, I. AND R. M. FRAPS: *Endocrinology* **44:** 134. 1949.
3. ROTHCHILD, I. AND R. M. FRAPS: *Endocrinology* **44:** 141. 1949.
4. FRAPS, R. M.: *Proc. Nat. Acad. Sci. Wash.* **40:** 348. 1954.
5. WARREN, D. C. AND H. M. SCOTT: *Poult. Sci.* **14:** 195. 1935.
6. HILL, R. T. AND A. S. PARKES: *Proc. Roy. Soc.* (*B*). **115:** 402. 1934.
7. KUPPERMAN, H. S., W. H. ELDER AND R. K. MEYER: *Endocrinology* **29:** 23. 1941.
8. GREEP, R. O., H. L. FEVOLD AND F. L. HISAW: *Anat. Rec.* **65:** 261. 1936.
9. LADMAN, A. J. AND M. N. RUNNER: *Endocrinology* **48:** 358. 1951.
10. LEVIN, L. AND H. H. TYNDALE: *Amer. J. Physiol.* **119:** 360. 1937.
11. BURDICK, H. O., H. WATSON, V. CIAMPA AND T. CIAMPA: *Endocrinology* **33:** 1. 1943.
12. KIRKHAM, K. E.: Ph.D. Thesis. Univ. Lib. Cambridge. 1957.
13. EMMENS, C. W.: In *Hormone Assay*, Academic Press: N. Y. 1950.
14. FINNEY, D. J.: In *Statistical Method in Biological Assay*. London: Griffin. 1955.
15. GADDUM, J. H.: In *Polypeptides which Stimulate Plain Muscle*. Edinburgh: Livingstone. 1933.
16. LORAINE, J. A.: *Clinical Application of Hormone Assay*. Edinburgh: Livingstone. 1958.
17. VAN TIENHOVEN, A., A. V. NALBANDOV AND H. W. NORTON: *Endocrinology* **54:** 605. 1954.
18. BROWN, P. S.: *J. Endocrin.* **13:** 59. 1955.
19. GREEP, R. O., H. B. VAN DYKE AND B. F. CHOW: *Endocrinology* **30:** 635. 1942.
20. LORAINE, J. A. AND J. B. BROWN: *Acta Endocr. Copenhagen* **17:** 25. 1954.

PREVENTION OF INSULIN-I^{131} ADSORPTION TO GLASS

ABSTRACT

It has been shown that human serum albumin in a final concentration of 0.5 mg./ml. can effectively prevent insulin-I^{131} from being adsorbed to glass surfaces. Of the insulin-I^{131} introduced to the system 95.8% was recoverable when precipitated with 20% trichloroacetic acid after a period of incubation for 30 minutes when protected by this concentration of albumin. It appears that human serum albumin can be reliably employed to protect insulin-I^{131} when dilutions of standards or working solutions are made for radioactivity measurements.

Radioactive proteins have been shown to be adsorbed to glass surfaces by several investigators (1–6). Newerly and Berson (2) have reported that these labeled proteins adsorb non-specifically to nearly every inert surface. More specifically, materials reportedly adsorbing radioactive proteins are pyrex glass containers, siliconized glassware and polyethylene reagent bottles (3). Magnus *et al.* (7) demonstrated the loss of activity of tuberculin preparations in such materials as quartz, nylon or silicone-treated ampules. When measuring the radioactivity of less than microgram quantities of labeled proteins having a high specific activity, such as encountered when preparing standards and working solutions, the loss of radioactivity to the vessel becomes a prime consideration.

Some agents appearing in the literature that protect radioactive proteins against adsorption to glass are 5% gelatin (3, 8), plasma albumin (4) and bovine albumin (1). Williams *et al.* (5) stated that the loss of radioactivity by adsorption could be minimized by addition of several milligrams of protein. However, there are no data in the literature concerning the *minimum* concentration of added protein necessary to prevent the loss of radioactive proteins to glass surfaces. The work herein presented outlines a simple and convenient quantitative method of measuring the loss of insulin-I^{131} to glass surfaces, and establishes a minimum effective level of added proteins at which the insulin-I^{131} can be protected from adsorption to glass.

METHODS AND MATERIALS

Insulin-I^{131}[1] was dialyzed at 2–5° C against 0.01M sodium phosphate buffer, pH 7.4, for about 18 hours, and then appropriately diluted with 0.01M sodium phosphate buffer. Stock human serum albumin (250 mg./ml.)[2] was diluted with 0.25M sucrose-0.1M sodium phosphate, pH 7.6. All glassware and reagents were maintained at 2–5° C.

One ml. of diluted HSA, and one ml. of dialyzed insulin-I^{131} were placed in a 20 ml. glass beaker. The concentration of the insulin ranged from 0.87×10^{-2} μg./ml. to 1.32×10^{-2} μg./ml. The radioactivity of insulin-I^{131} working solution was 0.04 (0.035–0.037) microcuries per ml. One ml. of 0.25M sucrose-0.1M sodium phosphate buffer, pH 7.6, plus one ml. of insulin-I^{131} were used for the "O" samples. Samples were incubated in a Dubnoff metabolic incubator at 85 excursions per minute for 30 minutes at 37° C. At the termination of 30 minutes 0.2 ml. (50 mg.) of stock HSA was quickly added to each sample to facilitate precipitation (5). Samples were then immediately poured into six ml. of 20% trichloracetic acid (TCA). Each beaker was rinsed twice with a total of about one ml. of distilled water and then inverted to drain for 15 minutes at room temperature. The wash was combined with the appropriate samples. The TCA precipitates, containing the precipitable insulin-I^{131}, were collected by centrifugation. The TCA supernatnants, representing the non-precipitable radioactivity, were decanted and diluted for counting. The TCA precipitated pellets were washed one time with 5% TCA, centrifuged, and the second supernatants were discarded. The TCA precipitated pellets were digested with 30% KOH and suitable aliquots were taken for counting.

For recovery of the adsorbed radioactivity, four ml. of 30% KOH, which facilitated removal of the adsorbed insulin-I^{131} (1), was added to each beaker, and the beakers were returned to the Dubnoff for five minutes. The total volume of the beaker wash was decanted into vials for counting.

Four ml. aliquots of all samples were placed in screw-capped vials and counted in a well type scintillation counter attached to a conventional scaler.

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[1] Obtained from Abbott Laboratories, Oak Ridge, Tennessee.

[2] The human serum albumin, abbreviated HSA, was obtained from Cutter Laboratories, Berkeley, California. The HSA can be outdated material provided it is kept sterile and refrigerated.

TABLE 1. EFFECT OF HUMAN SERUM ALBUMIN (HSA) ON ADSORPTION OF INSULIN-I^{131} TO GLASS

Final concentration of HSA (mg./ml.)	Number of samples	Per cent recoverable radioactivity*		
		TCA precipitate	TCA supernatant	30% KOH beaker wash
0	20	68.7 ± 2.33**	5.6 ± 0.50**	20.9 ± 1.95**
0.1	10	89.2 ± 1.43	3.3 ± 0.25	7.1 ± 1.01
0.5	10	95.8 ± 0.76	1.6 ± 0.06	3.3 ± 0.48
1.0	10	93.1 ± 1.01	1.4 ± 0.10	2.4 ± 0.19
5.0	9	95.8 ± 0.80	1.0 ± 0.07	2.1 ± 0.17
10.0	20	95.8 ± 0.56	1.3 ± 0.24	2.2 ± 0.18
25.0	10	94.7 ± 0.92	1.5 ± 0.06	2.4 ± 0.22
50.0	9	95.6 ± 0.80	1.7 ± 0.07	1.8 ± 0.20

* The per cent recoverable radioactivity values are means of values individually determined using the number of samples specified in the appropriate group.
** Standard error of the mean.

RESULTS AND DISCUSSION

From Table 1 it can be seen that a 20.9% loss of insulin-I^{131} by adsorption resulted in 30 minutes when no carrier protein was present. This is consistent with the findings of Hill (3) and Bull (6) of an initial rapid loss of radioactivity from solutions followed by a slower loss when insulin-I^{131} was diluted without accompanying protein. However, when 0.1 mg./ml. final concentration of HSA was introduced to the incubated sample, a nearly three-fold drop in adsorption to the glass was observed. Only 1.8% of the total radioactivity remained in the beaker when a 500 fold increase of the initial concentration of HSA was reached.

Further, one can see from these data that little increased protection of the radioactivity has been achieved by increasing the concentration of HSA above 0.5 mg./ml. Therefore, at this concentration it would appear from our results that 95.8% of the radioactivity can be reproducibly recovered, and that only 3.3% remained adsorbed to the wall of the vessel.

Hill (8) has been able to elute the adsorbed insulin-I^{131} having hypoglycemic activity by treating the container with 5% gelatin. Under our conditions the radioactivity was not readily removed by repeated washings with water. However, approximately 50% of the unprotected adsorbed radioactivity could be recovered when a solution containing 10 mg. HSA per ml. was incubated in the drained beakers. No attempt was made to demonstrate hypoglycemic activity of the insulin-I^{131} in our experiments. However, at the level of substitution of the commercially available hormone, 0.5–1.0 atom I per 6,000 molecular weight of insulin, full biological potency has been assured. No loss in biological activity should have resulted from our experiments since no inactivating system was present, and optimum physical conditions were closely maintained.

Therefore, it would appear to be useful to employ HSA in a final concentration of 0.5 mg./ml. to prevent losses of insulin-I^{131} by adsorption to glass surfaces when working with dilute solutions which may not contain other protein.

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REFERENCES

1. Ferrebee, J. W., B. B. Johnson, J. C. Mithoefer and J. W. Gardello: *Endocrinology* **48**: 277. 1951.
2. Newerly, K. and S. A. Berson: *Proc. Soc. Exper. Biol. & Med.* **94**: 751. 1957.
3. Hill, J. B.: *Endocrinology* **65**: 515. 1959.
4. Reeve, E. B. and J. J. Franks: *Proc. Soc. Exper. Biol. & Med.* **93**: 299. 1956.
5. Williams, R. H., J. S. Hay and M. B. Tjaden: *Ann. New York Acad. Sc.* **74**: 513. 1958–59.
6. Bull, H. B.: *Biochim. et biophys. acta.* **19**: 464. 1956.
7. Magnus, K., J. Guld, H. Waaler and M. Magnusson: *Am. Rev. Tuberc.* **74**: 297. 1956.
8. Hill, J. B.: *Proc. Soc. Exper. Biol. & Med.* **102**: 75. 1959.

STUDIES ON THE BIOLOGICAL ACTIVITY OF GONADOTROPIC EXTRACTS PREPARED FROM HUMAN POSTMENOPAUSAL URINE

ABSTRACT

Extracts prepared from human postmenopausal urine by the kaolin-acetone, tannic acid and attapulgite methods show similar ratios of FSH and ICSH activities. It is suggested that urinary extracts prepared by these methods can be assayed in terms of reference materials, e.g. HMG-20A and HMG-24, prepared by the kaolin-acetone method.

Most investigators now agree that pituitary tissue derived from man and from various animal species contains the two gonadotropic substances, follicle-stimulating hormone (FSH) and interstitial cell-stimulating hormone (ICSH). The presence of both FSH and ICSH activity has been demonstrated in human urine of both sexes and a various ages (1, 2, 3, 4) but, at the time of writing, a satisfactory separation of human urinary FSH from human urinary ICSH has not been achieved.

It has been shown that the biological activity of human pituitary gonadotropins (HPG) in urine can vary with the method of extraction employed (4, 5). The purpose of the present investigation was to obtain further information on the influence of extraction methods on the biological activity of urinary HPG. Three extraction methods were used and the material prepared by each technique was assayed for FSH and ICSH activity by tests generally assumed to be specific for these hormones. In this way it was possible to study the effect of the extraction method on the ratio of these two activites.

MATERIALS AND METHODS

1. *Urine collections.* Postmenopausal urine was collected both from ambulant and from hospitalized subjects. Two separate pools of urine were studied, designated henceforth *pool 1* and *pool 2*. The urine was stored in a cold room at 4° C and all extractions were performed within one week of collection.

2. *Extraction methods.* These were as follows:

(a) The kaolin-acetone method of Loraine and Brown (6). In this technique the tricalcium phosphate purification procedure was omitted.

(b) The tannic acid precipitation method of Johnsen (7).

(c) The attapulgite adsorption method of Salhanick (8).

Five liters of urine were extracted by each method. Pool 1 was extracted by methods (b) and (c) only while in pool 2 all 3 methods were used.

3. *Bioassay methods.* These were as follows:

(a) Increase in ovarian weight in intact immature mice treated with an excess of human chorionic gonadotropin (HCG)—"augmentation test" (9). This test measures mainly FSH activity.

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TABLE 1. COMPARISON OF EXTRACTION METHODS—POOL 1

Method of extraction	Bioassay method	Design of assay	No. of animals	Index of precision λ	Result HMG units/liter	Fiducial limits (P =0.95) HMG units/liter
Tannic acid precipitation	Augmentation test	2+2	14	0.13	99	68–151
	Hypophysectomised rat prostate test	2+2	15	0.13	88	63–131
	Mouse uterus test	2+1	11	0.05	85	98–137
Attapulgite adsorption	Augmentation test	2+2	14	0.18	83	47–141
	Hypophysectomised rat prostate test	2+2	15	0.13	77	55–108
	Mouse uterus test	2+1	10	0.05	62	60– 87

(b) Increase in ventral prostatic weight in hypophysectomized immature rats (10). This test is generally assumed to be specific for ICSH activity.

(c) Increase in uterine weight in intact immature mice (10). This method is not specific for either FSH or ICSH but provides an index of the "total gonadotropic activity" of urinary extracts.

4. *Design of assays.* In all, 15 assays were performed, by each of the three methods. Results were expressed in terms of the international reference preparation HMG-24, as HMG units/liter of urine. In all instances in which the augmentation and hypophysectomized rat prostate tests were used the design was a 4-point assay with two doses of the reference material (S) and unknown (U) preparations respectively, and with equal spacing of the log doses. With the mouse uterus test assays on pool 2 were also of a 4-point design but in pool 1 a 3-point design with 2 doses of S and one of U was employed. In the augmentation and hypophysectomized rat prostate tests the log dose interval was equal to $\log_{10}3$ (0.477) while in the mouse uterus test the corresponding figure was $\log_{10}2$ (0.301). Total dosages per animal of HMG-24 used in constructing the dose-effect curves were for the augmentation test 1.0 and 3.0 mg.; for the hypophysectomized rat prostate test 2.2 and 6.6 mg. and for the mouse uterus test 0.9 and 1.8 mg. With pool 1 the extracts were administered in total doses per animal equivalent to 12.0 and 36.0 ml. of the original urine in the case of the augmentation test and to 30.0 and 90.0 ml. in the case of the hypophysectomized rat prostate test. With pool 2 the corresponding figures were respectively 5.0 and 15.0 ml. and 13.0 and 39.0 ml. When 4-point assays were conducted by the mouse uterus test on pool 2 the total dose-equivalents of original urine per animal were 5.0 and 10.0 ml.

The methods used in performing validity tests and in calculating the indices of precision, relative potencies and limits of error, have been described elsewhere (11, 12). It should be emphasized that in no assay did significant deviation for parallelism occur. The index of discrimination (13) was calculated by dividing the result obtained by one method by that obtained by another.

RESULTS

These are shown in Tables 1 and 2. In all assays the degree of precision was satisfactory, the figure for "λ" being below 0.20. The mean index of precision for the five assays performed by the augmentation test was 0.14 and the corresponding figures for the hypophysectomized rat prostate and mouse uterus tests were respectively 0.13 and 0.05.

Tables 1 and 2 indicate that the gonadotropic activity of pool 1 was considerably lower than that of pool 2. In pool 1 the lowest figure for HPG concentration was 62 HMG units/liter and the highest 99 HMG units/liter; the corresponding figures for pool 2 were respectively 149 and 247 HMG units/liter.

TABLE 2. COMPARISON OF EXTRACTION METHODS—POOL 2

Method of extraction	Bioassay method	Design of assay	No. of animals	Index of precision	Result HMG units/liter	Fiducial limits (P =0.95) HMG units/liter
Tannic acid precipitation	Augmentation test	2+2	15	0.13	247	176–362
	Hypophysectomised rat prostate test	2+2	20	0.14	197	166–317
	Mouse uterus test	2+2	15	0.07	178	158–201
Attapulgite adsorption	Augmentation test	2+2	16	0.13	198	140–278
	Hypophysectomised rat prostate test	2+2	19	0.13	149	109–199
	Mouse uterus test	2+2	20	0.05	178	160–199
Kaolin-acetone	Augmentation test	2+2	16	0.15	209	140–316
	Hypophysectomised rat prostate test	2+2	16	0.10	197	154–262
	Mouse uterus test	2+2	18	0.05	178	158–201

TABLE 3. INDICES OF DISCRIMINATION FOR VARIOUS COMBINATIONS OF TESTS

Material tested	Method of extraction	Index of discrimination		
		Ovary (mouse)/ prostate (rat)	Prostate (rat) /uterus (mouse)	Ovary (mouse) /uterus (mouse)
Pool 1	Tannic acid precipitation	1.12	1.04	1.16
Pool 1	Attapulgite adsorption	1.08	1.24	1.34
Pool 2	Kaolin-acetone	1.06	1.11	1.17
Pool 2	Tannic acid precipitation	1.25	1.11	1.39
Pool 2	Attapulgite adsorption	1.33	0.84	1.11

The data in columns 6 and 7 show that in both pools the HPG concentrations obtained by different extraction methods and by different biological tests agreed reasonably well. In particular, it should be noted that in all assays on pool 2 and in 5 out of the 6 assays on pool 1 there was complete overlap of the fiducial limits of error.

The indices of discrimination (12) obtained from the various combinations of assays are shown in Table 3. It will be noted that all values closely approximate to unity indicating that the materials prepared by the three extraction methods are qualitatively similar. The figures in column 3 provide an estimate of the ratio of FSH to ICSH activity in the various urinary extracts; it is apparent that the method of extraction of the urine had little or no effect on this ratio.

DISCUSSION

The present investigation has shown that extracts prepared from postmenopausal urine by the kaolin-acetone, tannic acid and attapulgite methods are qualitatively similar and that, when these extracts are assayed by appropriate biological tests, the ratio FSH activity/ICSH activity remains approximately the same. This fact is of importance in clinical assays for HPG where an extract prepared by one method may have to be assayed against a standard prepared by another. The data reported herein support the view that material prepared by the three extraction methods studied can justifiably be assayed against reference preparations such as HMG-24 and HMG-20A which are prepared by the kaolin-acetone method. It must be emphasized, however, that this conclusion applies only to the three extraction methods which have formed the basis of this study, for it has already been shown that material prepared in other ways e.g. by the zeolite (permutit) method (4), is qualitatively different from HMG-20A.

The evidence from the present study is at variance with the conclusions of Walter (5) who, using the mouse uterus and hypophysectomized rat prostate tests, found that extracts prepared by the tannic acid method were qualitatively different from those prepared by the kaolin-acetone method. The reason for this discrepancy is not clear but may be explained on a methodological basis. In Walter's experiments the tannic acid method used was that of Levin and Tyndale (14) while in the present study the method of Johnsen (7) was employed. These two extraction procedures are by no means identical and it is possible that because of differences in technique, the final products obtained are qualitatively dissimilar.

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REFERENCES

1. McArthur, J. W.: *Endocrinology* **50:** 304. 1952.
2. Loraine, J. A. and J. B. Brown: *J. Clin. Endocrinol. & Metab.* **16:** 1180. 1956.
3. Albert, A., R. Borth, E. Diczfalusy, J. A. Loraine, B. Lunenfeld, J. W. McArthur and E. Rosemberg: *J. Clin. Endocrinol. & Metab.* **18:** 1117. 1958.
4. Benz, F., R. Borth, P. S. Brown, A. C. Crooke, J. B. Dekanski, E. Diczfalusy, J. A. Loraine, B. Lunenfeld and W. Schuler: *J. Endocrin.* (British). **19:** 158. 1959.
5. Walter, K.: *J. Endocrin.* (British). **15:** 119. 1957.
6. Loraine, J. A. and J. B. Brown: *J. Endocrin.* (British) **18:** 77. 1959.
7. Johnsen, S. G.: *Acta endocr. Copenhagen* **28:** 69. 1958.
8. Salhanick, H. A. In "Proceedings of Workshop Meeting on Gonadotropins," ed. A. Albert, 1960. *in press.*
9. Brown, P. S.: *J. Endocrin.* (British) **13:** 59. 1955.
10. Loraine, J. A. and J. B. Brown: *Acta endocr. Copenhagen* **17:** 250. 1954.
11. Finney, D. J.: Statistical Methods in Biological Assay. Charles Griffin, London, 1952.
12. Gaddum, J. H.: *Pharm. Rev.* **5:** 87. 1953.
13. Gaddum, J. H.: Polypeptides which Stimulate Plain Muscle. Livingstone, Edinburgh, 1955. p. 133.
14. Levin, L. and H. H. Tyndale: *Proc. Soc. exp. Biol.* N. Y. **34:** 516. 1936.

THE ENDOCRINE SOCIETY

The 1961 Annual Meeting

The Forty-Third Annual Meeting of the Endocrine Society will be held in the Hotel Biltmore, New York, N. Y., Thursday, Friday and Saturday, *June 22, 23 and 24, 1961.*

The Scientific Sessions will be held from 9:00 A.M. to 5:00 P.M. daily and in addition there will be simultaneous afternoon sessions. The annual dinner is scheduled for Friday, June 23, at 7:30 P.M., preceded by cocktails at 6:30 P.M.

All members are urged to make their hotel reservations immediately. The Biltmore will hold 300 bedrooms for members until May 1, 1961, after which time the hotel will not guarantee further reservations. Therefore it is imperative that you make your reservations early, directly with the hotel, advising them of time and date of arrival and departure. If you plan on remaining through June 26 for the American Medical Association and other meetings, it is imperative that your reservations be made from June 26 on directly through the A.M.A. Housing Bureau. Make your reservations early and avoid disappointment. Hotel reservation card will be mailed to members shortly after January 1, 1961.

Final program, membership card and advance registration forms will be sent on May 1, 1961 to members whose current dues have been paid.

Those wishing to present papers, which will be strictly limited to ten minutes, should send an original and four copies of the title and abstract to the Vice-President, Dr. Roy Hertz, National Cancer Institute, Bethesda, Maryland, not later than February 15, 1961. It is imperative that the abstracts be informative and complete with results and conclusions —not a statement that those will be presented at the meeting—in order that they may be of reference value and suitable for printing in the program. The reading and processing of approximately two hundred abstracts submitted each year and compiling of the program from these involves a tremendous amount of time and effort on the part of the Program Committee.

THE COUNCIL REQUESTS THAT AUTHORS ADHERE STRICTLY TO THE FOLLOWING RULES WHEN SUBMITTING ABSTRACTS, OTHERWISE THEY CANNOT BE CONSIDERED:

1. IT IS ASSUMED THAT ABSTRACTS SUBMITTED FOR THIS PROGRAM HAVE NOT AND WILL NOT BE SUBMITTED ELSEWHERE.
2. Abstracts may not exceed two hundred words, or equivalent space

exclusive of title. No footnotes or acknowledgements to sponsors can be published. Reference, if used, must be placed in the body of the text. The abstract should consist of a single paragraph, if possible. Structural chemical formula cannot be used.

3. The title heading must be arranged as follows:
   Line 1. Title, not to exceed fifteen words;
   Line 2. Author/s. The name of each non-member author collaborating with member-authors is to be followed by the phrase "(by invitation)". Names of non-members who are introduced, i.e., who are not collaborators with member-authors, are to be followed by the phrase "(introduced by . . .)". The principal degree, e.g., M.D., of each author should be written after his name.
   Line 3. Institution of origin and city in which institution is located.

1961 LAURENTIAN HORMONE CONFERENCE

THE 1961 Laurentian Hormone Conference of the AAAS will be held at Hoberg's Resort, Lake County, California, during the week September 4–9. Investigators interested in attending this Conference should make application to the Committee on Arrangement of the Laurentian Hormone Conference, 222 Maple Ave., Shrewsbury, Massachusetts, at an early date and in any event no later than May 10, 1961. A conference rate of $14.50 per day per person is extended to all invited participants, with reduced rates for children. Since the number of our participants is necessarily limited by available accommodations, all applications are screened and invitations to attend are issued by the Committee on Arrangements by the second week in June. Invitations issued to applicants will include family members and indication of the number of such members should be made upon application to the Committee. The following program has been arranged.

1961 LAURENTIAN HORMONE CONFERENCE PROGRAM

SEPTEMBER 4 THROUGH 9

I. HORMONAL POLYPEPTIDES AND THEIR FUNCTIONAL ACTIVITIES

Isolation, structure, synthesis and biological activity of adrenocorticotropins (ACTH).
C. H. Li, University of California, Berkeley.
Monday evening, September 4

Synthetic pituitary hormones.
Klaus Hofmann and Haruaki Yajima, University of Pittsburgh School of Medicine, Pittsburgh.
Tuesday morning, September 5

Studies on a lipid-mobilizing substance of the pituitary gland.
Daniel Rudman, Robert L. Hirsch, and Forrest E. Kendall; Columbia University Research Service, Goldwater Memorial Hospital, and Departments of Medicine, Pathology and Biochemistry, College of Physicians & Surgeons, New York.
Tuesday morning, September 5

Normal and abnormal regulation of corticotropin secretion in man.
Grant W. Liddle, Donald Island, and Clifton Meader; Vanderbilt University, Nashville.
Tuesday evening, September 5

Angiotensin, a renal hormone.
Irvine H. Page and F. M. Bumpus, The Cleveland Clinic, Cleveland.
Wednesday morning, September 6

II. THYROID HORMONES

The natural occurrence of goitrogenic agents.
Monte A. Greer, University of Oregon School of Medicine, Portland.
Wednesday morning, September 6

Some extracellular and intracellular mechanisms governing the utilization of thyroid hormones.

Jamshed R. Tata, Wenner-Grens Institute, Stockholm, Sweden.
Wednesday evening, September 6

III. Parathyroid Hormone

The chemistry, biochemistry, and physiology of the parathyroid hormone.
Howard Rasmussen, Rockefeller Institute for Medical Research, New York.
Thursday morning, September 7

Clinical endocrinology of parathyroid hormone excess.
Gilbert S. Gordan, Eugene Eisenberg and H. F. Loken; University of California Medical Center, San Francisco.
Thursday morning, September 7

IV. Steroid Hormones

Methods for measuring estrogens and their application to problems in gynecology and obstetrics.
J. B. Brown and G. D. Matthew, Clinical Endocrinology Research Unit, Edinburgh, Scotland.
Friday morning, September 8

Some studies of the mechanism of estrogen action.
Elwood V. Jensen, Ben May Laboratories, University of Chicago, Chicago.
Friday morning, September 8

Anti-estrogens.
C. W. Emmens, Department of Veterinary Physiology, Sydney, Australia.
Friday evening, September 8

The use of cortisone to condition hosts for successful tissue grafting.
Helene Wallace Toolan, Sloan-Kettering Institute for Cancer Research, New York.
Saturday morning, September 9

A comparison of the mechanisms of hormonal and substrate induction of rat liver tryptophan pyrrolase.
Philip Feigelson, M. Feigelson and O. Greengard; Columbia University Institute of Cancer Research, New York.
Saturday morning, September 9

FIRST EUROPEAN COLLOQUE ON ENDOCRINOLOGY

The First European Colloque on Endocrinology, organized by the "Association Médicale France-Espagne" and the Medical School of Barcelona, will be held from 20th. to 22nd. May, 1961 (Whitsuntide), in the Park-Hotel San Jorge, on the "Costa Brava," some 70 kms. north of Barcelona.

The Conference will consist of a series of lectures on clinical, experimental and biochemical endocrinology, which will be delivered by eminent scientists from various European countries. After each lecture there will be a brief discussion, during which short communications on the specific subject will be accepted.

A limited number of rooms have been reserved in the Hotel in which the Conference will be held for those who first indicate that they will attend. Full board and lodging, including taxes, is only pesetas 300 per head per day, which is equivalent to $5 U.S.A.

All inquiries and request to join the meeting should be addressed to Dr. Joseph M. Cañadell, Avenida Generalísimo 497, Barcelona, 15, Spain.